Functional Genetic Mapping of Pheudomonas Aeruginosa from Cystic Fibrosis Lungs

Yang, Lei

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GENETIC ADAPTATION OF *PSEUDOMONAS AERUGINOSA* IN CYSTIC FIBROSIS PATIENTS

PH.D. THESIS

LEI YANG

Centre of System Microbiology

Department of Systems Biology

Technical University of Denmark

Nothing in Biology Makes Sense Except in the Light of Evolution.

— *Theodosius Dobzhansky 1973*

Preface

This thesis is submitted as a partial fulfilment of the requirements to obtain the Ph.D. degree in Technical University of Denmark (DTU). The work presented was carried out in the Infection Microbiology Group, Centre of System Microbiology, Department of Systems Biology in DTU under the supervision of Prof. Dr. Søren Molin. The work was performed from January 2007 to December 2009 and the thesis was submitted at the end of March 2010. This Ph.D. project was co-financed by DTU and Danish Research Council.

Lei Yang 杨蕾
Kgs. Lyngby
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Besides all the people who have been kind and helpful, I would like to thank DTU and Danish Research Council for financially supporting my Ph.D. study. I am also grateful for Oticon Foundation and Otto Mønsted Foundation for travel grants for conferences and workshops.

Summary

The evolutionary success can be simply described as persistence of an organism in its environment. This thesis is a story of bacterial adaptation and persistence in chronic infection.

The ability of bacterial pathogens to colonize and establish lifetime persistence in the host is a fundamental problem for infectious disease control. Very often this persistence is a procedure of adaptation of pathogens driven by selective forces imposed by the host environment. Despite many years of intensive research, we have very limited knowledge about the basis of this process. Experimental evolution using microorganisms has allowed us to unravel dynamics and genetic details of adaptation when bacteria were propagated to replicate for many generations in a novel environment. However, these investigations were mainly performed with isolated pure cultures evolving under defined conditions. It is extremely interesting to uncover the mechanisms of bacterial adaptation in 'real-world' disease cases, and assess the compatibility of these with current evolutionary theories drawn from experimental evolution studies.

Our model system is *Pseudomonas aeruginosa* chronic infection in cystic fibrosis (CF) patients. CF is a genetic disorder that mainly leads to deficient mucociliary clearance and microbial colonization in the airway mucus. The ubiquitous bacterium *P. aeruginosa* is the most prevalent pathogen in CF patients and to a large extent responsible for decreased lung function. A unique collection of *P. aeruginosa* isolates from CF patients in the Copenhagen CF centre initiated since 1973 allowed us to follow the adaptation process of this bacterium in CF airways for over 35 years.

Typically, CF patients acquire *P. aeruginosa* from the environment and the bacteria persist in the airway through genetic adaptation. Compared to environmental isolates or standard lab strains, *P. aeruginosa* strains isolated from CF patients showed reduced growth rates as the infection progresses. Using fluorescent *in situ* hybridization (FISH) we could estimate the *in vivo* growth activity of *P. aeruginosa* in the CF airway by measuring the ribosome content. Surprisingly the majority of the cells are actively growing and the average *in situ* doubling time is between 100-150 min. This ensured high turn-over rate of biomass and enormous possibilities for genetic changes.

One genotype that we have focused is the b clone that has been dominant and transmissible in many chronic patients. Since its first appearance in 1973 this clone has evolved in different patients for more than 150,000 generations. Since the human airway is a complex environment, intuitively one would imagine that adaptation would create diversity. However, adapted b strains isolated from different patients have remarkable similarity when we measured the transcriptome and catabolic functions. We also found that very few mutations in the evolution of b clone significantly enhanced the adaptation and allowed it to successfully sweep across many patients. Those important mutations occurred very early in the global regulators and have great impact on the overall pattern of expression levels and phenotypes. These findings resemble the evolution trajectory concluded from experimental microbial evolution studies. Besides, parallel evolution traits have also been observed, in which similar phenotypic changes occurred through independent genetic alterations. The overall result of the evolution of *P. aeruginosa* in CF airway for the b genotype is that it drifted away from the ancestor in terms of genetic content and various phenotypes, directing the consequence of creating specialists good at reproducing in the stressful environment in human CF lung. At the same time, the adapted strain seems to behave like a generalist capable of occupying all different niches in the lung, since we did not observe an increase of diversity during the evolution.

Dansk Resumé

Patogene bakteriers evne til at kolonisere og etablere livsvarig infektion i værten er et grundlæggende problem for bekæmpelsen af infektionssygdomme. Et eksempel på bakteriers vedholdenhed i værten ses ved kronisk infektion med bakterien *Pseudomonas aeruginosa* (*P. aeruginosa*) i cystisk fibrose (CF) patienter, som opnås gennem genetisk tilpasning til miljøet i CF patienters luftveje. Siden 1973 er *P. aeruginosa* isolater fra CF patienter tilknyttet Københavns CF center blevet gemt i en unik samling, hvilket har gjort os i stand til at følge bakteriens tilpasningsproces til CF luftvejene over en periode på mere end 35 år.

CF patienterne bliver typisk inficeret med *P. aeruginosa* fra miljøet, hvorefter bakterierne forbliver i luftvejene vha. genetiske adaptationer. *P. aeruginosa* isolater fra CF patienter vokser langsommere end isolater fra miljøet eller standard laboratorie-stammer og efterhånden som infektionen skrider frem bliver denne forskel endnu mere markant. Vi har anvendt fluorescent in situ hybridization til at bestemme mængden af ribosomer i *P. aeruginosa* prøver fra CF patienter, hvorudfra vi kan estimere *in vivo* væksthastigheden for *P. aeruginosa* i CF luftvejene. Overraskende viste det sig, at størstedelen af cellerne er aktive og vokser med en gennemsnitlig *in situ* fordoblingstid på 100-150 min. Som følge heraf må der være en høj omsætningshastighed af biomasse i CF luftvejene og et enormt potentiale for genetiske ændringer.

Vi har fokuseret på en genotype kaldet den b klon, som har været dominerende og er blevet overført mellem mange kronisk inficerede patienter. Den b klon blev opdaget første gang i 1973 og har indtil nu udviklet sig i forskellige patienter i mere end 150.000 generationer. Som følge af det komplekse miljø, som de humane luftveje udgør, kunne man umiddelbart forvente at tilpasning til luftvejene ville resultere i diversitet. Imidlertid har vi observeret, at adapterede b stammer isoleret fra forskellige patienter udviser påfaldende ligheder i deres transkriptom og kataboliske kapacitet. Ydermere har vi for udviklingen af den b klon set, at fåtal af mutationer er ansvarlige for meget af adaptationen af den klon og at disse mutationer har gjort klonen i stand til at sprede sig til mange patienter. Mutationerne opstod tidligt i forløbet i gener kodende for globale regulatorer og har stor indvirkning på transkriptomet og stammernes fænotyper. Disse resultater minder om de evolutionsforløb, som er blevet observeret under eksperimentelle evolutionsstudier med mikroorganismer. Derudover er der også blevet observeret parallel evolution af

karakteristika, hvor forskellige genetiske ændringer har medført ens fænotypiske ændringer. Overordnet gælder der for udviklingen af *P. aeruginosa* i CF luftvejene for den b genotype, at udviklingen mod specialister, som er gode til at overleve i den humane CF lunges stressende miljø, har medført, at den b genotype er afveget fra stamfaderen i forhold til arvemasse og forskellige fænotyper.

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Chapter 1 Introduction

1.1 *Pseudomonas aeruginosa* - an opportunistic pathogen

1.1.1 *P. aeruginosa* – a generalist in different environments

Pseudomonas aeruginosa is a motile rod-shaped Gram-negative bacterium (**Figure 1**) belonging to the genus *Pseudomonas*, which consists of several species of environmental bacteria. It is ubiquitous in soil and water, and able to colonize plants, insects and mammals. It does not grow fermentatively but is capable to grow anaerobically using nitrate or arginine as respiratory electron acceptors. *P. aeruginosa* grows as high as 42°C but optimally at 37°C, which is a feature enabling it to become the only animal pathogen in *Pseudomonas* genus. In human beings, *P. aeruginosa* is an opportunistic pathogen infecting immunocompromised patients, such as those with chronic obstructive pulmonary disease, severe burns and in intensive care units. *P. aeruginosa* can either invade tissues and blood and cause acute infections like bacteraemia, or colonize mucosal surface and develop chronic infections like in cystic fibrosis (CF) patients. In a surveillance study of patients in the intensive care unit in Europe, *P. aeruginosa* was accountable for 30% of pneumonias, 19% of urinary track infections, and 10% bloodstream infections (Spencer, 1996). *P. aeruginosa* has been ranked in the top 3 pathogens in human beings due to its wide existence in the environment and extreme difficulties in medical treatment. Therefore, it is of great importance to understand its pathogenicity and mechanisms of counteracting antimicrobial therapies.

The ability of *P. aeruginosa* to thrive in very diverse ecological niches and switch life styles quickly upon changing conditions is consistent with its relatively large genome size and the complex regulatory networks. The first *P. aeruginosa* genome was sequenced in 2000 using the strain PAO1, which is 6264,404 base pair and has 5570 open reading frames (ORFs) (Stover *et al.*, 2000). This genome size is comparable to the simple eukaryote *Saccharomyces cerevisiae* which has a genome encoding 6200 proteins (Ball *et al.*, 2000). Among the around 50% ORFs that have been assigned functional classes, PAO1 carries 517 genes encoding proteins with motifs of transcriptional regulators or two-component regulatory systems and 558 transporters

of small molecules, which corresponds to 9.3% and 10.0% of the genome respectively (Kulasekara & Lory, 2004). This large fraction of regulators and transporters further reflects its capability to respond to various stimuli and survive in different environments. To date, there are 7 *P. aeruginosa* genomes available in the National Center for Biotechnology Information (NCBI) genome database.

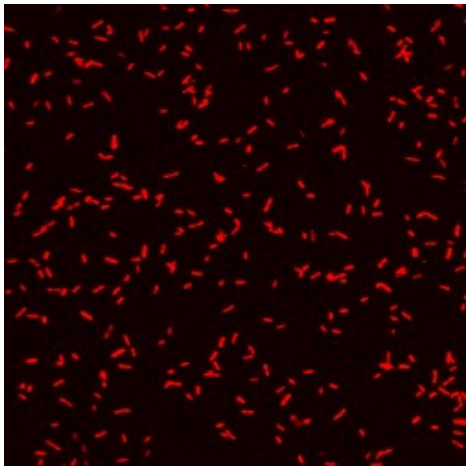


Figure 1. Fluorescent *in situ* hybridization (FISH) staining of *P. aeruginosa* culture with probes targeting 16S RNA (This work).

1.1.2 The mechanisms of antibiotic resistance by *P. aeruginosa*

P. aeruginosa is one of the 'superbugs' that can be multi-resistant to various antibiotics. The molecular mechanisms of resistance include efflux pumps, mutation on targets of antibiotics, membrane modification, expression of enzymes that destruct the drugs, *etc.* The efflux pump systems are secondary transporters or ATP-Binding Cassette (ABC) transporters that export a variety of agents including antibiotics, biocides, dyes, organic solvents, detergents, *etc*. *P. aeruginosa* has relatively high intrinsic antibiotic resistance due to efflux of the drugs (Li *et al.*, 2004). Based on amino acid identity, bacterial efflux pumps are categorized into five superfamilies: the ABC family, the resistance-nodulation-division (RND) family, the small multidrug resistance family, the major facilitator superfamily and the multidrug and toxic compound extrusion family (Lister *et al.*, 2009).

P. aeruginosa has efflux systems from all five superfamilies with largest number of predicted pumps belonging to the RND family (Stover et al., 2000). RND efflux pump systems are the most important family because they are associated with multi-resistance of *P. aeruginosa* to a variety of clinically relevant antibiotics. The characteristics of all twelve RND efflux pumps in *P. aeruginosa* are listed in Table 1.

Two of them are divalent metal cation transporters (CzrCBA and products of *PA2525-PA2528*). Efflux pump systems have different substrate spectrums with overlaps and they also differ in preferences of the same antibiotic class. For example, both MexAB-OprM and MexCD-OprJ can export β-lactams but MexCD-OprJ has a more narrow substrate profile of only the fourth-generation cephalosporins like cefepime (Lister et al., 2009).

RND pumps typically contain a cytoplasmic membrane transporter (RND), a periplasmic membrane fusion protein (MFP) and an outer membrane factor (OMF). Figure 2 illustrates the structure of a three-component RND efflux pump system, MexAB-OprM. The pump (MexB, RND) lies in the cytoplasmic membrane and the exit portal (OprM, OMF) is located in the outer membrane. They are linked via a lipoprotein (MexA, MFP). The efflux of drugs is coupled with the influx of proton.

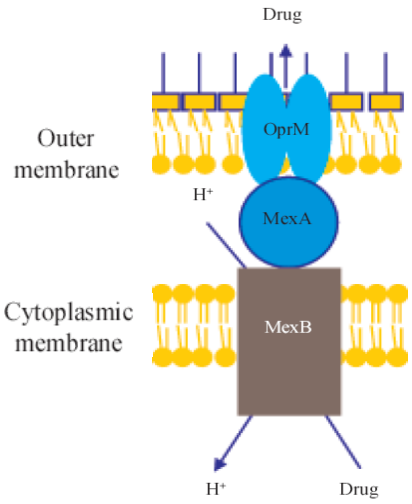


Figure 2. Structure of MexAB-OprM (Schweizer, 2003). The pump MexB (in the cytoplasmic membrane) is linked to the exit portal OprM (in the outer membrane) through lipoprotein MexA. The efflux of drugs is coupled with the influx of H^+.

Details of antibiotics resistance conferred by efflux pumps are described in following sections, however, one should know that efflux pumps are more than just resistance. They actually play other physiological roles that have great clinical relevance (Piddock, 2006). For example, in *P. aeruginosa* MexAB-OprM is the major player of intrinsic resistance (Poole *et al.*, 1993) and this system has very broad substrate spectrum (**Table 1**), including cell-produced quorum sensing (QS) signal homoserine lactones (HSL) (see **section 1.1.3**). The expression of *mexAB-oprM* is growth phase dependent mediated by HSL (Sawada *et al.*, 2004), indicating the involvement of this pump in cell-to-cell communication. This might contribute to the

pathogenicity of *P. aeruginosa* since QS governs many of the virulence factors (see **section 1.1.4**). It has been shown that in contrast to the wild type strain, *P. aeruginosa* lacking MexAB-OprM could not invade epithelial cells or kill leukocytes deficient mice (Hirakata *et al.,* 2002). Chen *et al.* also described that MexAB-OprM can be activated in response to oxidative response sensed by MexR (Chen *et al.,* 2008).

Table 1. RND efflux pumps in *P. aeruginosa* (Lister et al., 2009)

Operon	Components	Function	Regulators	Substrates		Intrinsic resistance #
				Antibiotics	Additional compounds	
mexAB-oprM	MexA MexB OprM	MFP RND OMF	MexR* (-) NalD (-) NalC (-)	Fluoroquinolones, β-lactams, β-lactams inhibitors, tetracycline, chloramphenicol, macrolides, novobiocin, trimethoprom, sulfonamides	Biocides (e.g.triclosan), detergents (e.g. SDS), HSL, aromatic hydrocarbons	Yes
mexCD-oprJ	MexC mexD OprJ	MFP RND OMF	NfxB* (-)	Fluoroquinolones, β-lactams, tetracycline, chloramphenicol, macrolides, novobiocin, trimethoprom	Biocides (e.g.triclosan), detergents (e.g. SDS), aromatic hydrocarbons	No
mexEF-oprN	MexE MexF OprN	MFP RND OMF	MexT* (+) MexS (-) MvaT (-)	Fluoroquinolones, chloramphenicol, trimethoprom	Biocides (e.g.triclosan), aromatic hydrocarbons	No
mexXY	MexX MexY OprM/Opm¤	MFP RND OMF	MexZ* (-)	Aminoglycosides, fluoroquinolones, β-lactams, tetracycline, chloramphenicol, macrolides		Yes
mexJK	MexJ MexK OprM/OpmH	MFP RND OMF	MexL* (-)	Tetracycline, erythromycin	Biocides (e.g.triclosan)	No
mexGHI-opmD	MexG MexH MexI OpmD	? MFP RND OMF	SoxR* (+)	Fluoroquinolones	Vanadium	n.i.
mexVW	MexV MexW OpmE	MFP RND OMF	n.i.	Fluoroquinolones, chloramphenicol, Tetracycline,erythromycin		n.i.

mexPQ-opmE	MexP MexQ OpmE	MFP RND OMF	n.i.	Fluoroquinolones, tetracycline, chloramphenicol, macrolides	n.i.
mexMN	MexM MexN OprM	MFP RND OMF	n.i.	Chloramphenicol, thiamphenicol	n.i.
triABC	TriA TriB TriC OpmH	MFP MFP RND OMF	n.i.	Triclosan	n.i.
czrCBA	CzrB CzrA CzrC	MFP RND OMF	CzrS*(+) CzrR*(+)	Cadmium, zinc	n.i.
PA2525-PA2528	PA2528 PA2527 PA2526 PA2525	MFP RND RND OMF	n.i.	n.i.	n.i.

*, major regulators that are located adjacent upstream of the operons.
(+) and (-) indicate positive or negative regulation.
n.i., not identified
¤, MexXY can use OpmB, OpmG, OpmH and/or OpmI as OMFs.
HSL, homoserine lactones
#, If an efflux pump contributes to intrinsic resistance, it has impact on wild-type susceptibility when the encoding genes are disrupted.

1.1.2.1 P. aeruginosa resistance to β-lactams

β-lactams (cefepime, ceftazidime, piperacillin-tazobactam, aztreonam *ect.*) are the oldest but still widely used antibiotics. The most common mechanism to become resistant to β-lactams is to express AmpC β-lactamase. In *P. aeruginosa* AmpC β-lactamase is expressed in a basal level and can be induced by β-lactams to a certain level. This is the main contributor of inherent resistance to β-lactams (Livermore, 1995). The expression of *ampC* is positively regulated by a transcriptional regulator AmpR and repressed by AmpD, AmpDh2 and AmDh3 (Juan *et al.*, 2006, Kong *et al.*, 2005). Mutations in *ampD* homologues can lead to stepwise derepression of AmpC and high resistance to all β-lactams except carbapenems (Juan et al., 2006). Recently a new β-lactamase PoxB was discovered to be responsible for β-lactams resistance when AmpC is deficient (Kong *et al.*, 2005). Efflux pump MexAB-OprM is constitutively produced under normal lab conditions and provide impermeability of a wide range of antibiotics, including β -lactams. It has also been found in CF isolates that modification in penicillin binding proteins (PBPs) could lead to β-lactams-resistance (Godfrey *et al.*, 1981).

Carbapenems including imipenem, doripenem, meropenem, *etc*. belong to a class of β-lactams that are highly resistance to β-lactamases. *P. aeruginosa* carries a substrate-specific porin OprD that facilitates the uptake of basic amino acids and carbapenems (Trias & Nikaido, 1990b, Trias & Nikaido, 1990a). OprD-deficient mutant *P. aeruginosa* displayed significantly increase of resistance to carbapenems (Sakyo *et al.*, 2006).

1.1.2.2 P. aeruginosa resistance to aminoglycosides

Aminoglycosides (AGs) are a broad spectrum of antibiotics targeting ribosome and impairing bacterial protein synthesis (Davies *et al.*, 1965). *P. aeruginosa* can become resistant to AGs like tobramycin and gentamicin by modifying the drugs through enzymes, such as aminoglycoside phosphoryltransferase APH (Zeng & Jin, 2003), aminoglycoside acetyltransferase (AAC), aminoglycoside phosphoryltransferase (APT) and aminoglycoside nucleotidyltransferase (ANT) (Mingeot-Leclercq *et al.*, 1999); APH is located in the chromosome (encoded by PA4119) whereas the latter three families were found only in imported plasmids.

In additional to aminoglycoside-modifying enzymes, *P. aeruginosa* can gain resistance to AGs by methylation of the 16s rRNA. Currently 5 ribosomal methylatransferase enzymes (RmtA, RmtV, RmtC, RmtD, and ArmA) have been found in clinical *P. aeruginosa* isolates and they are encoded by genes associated with

mobile genetic elements (Galimand *et al.*, 2005, Lister et al., 2009, Wachino *et al.*, 2006a, Wachino *et al.*, 2006b, Yokoyama *et al.*, 2003).

Furthermore, aminoglycoside response regulator (encoded by PA2818_arr) is involved in tobramycin induced biofilm formation through c-di-GMP (Hoffman *et al.*, 2005). Induction of efflux pump MexXY-OprM is also involved in AGs resistance. The most frequently found AGs resistant clinical isolates contain a mutation in MexZ, which is a repressor of MexXY (Vogne *et al.*, 2004).

1.1.2.3 P. aeruginosa resistance to fluoroquinolones

Fluoroquinolones like ciprofloxacin are another group of commonly used antibiotics that prevent DNA unwinding and duplicating. Mutations in DNA gyrase (*gyrA, gyrB*) and topoisomerase (*parC, parE*) exibit increased minimum inhibitory concentration (MIC) of *P. aeruginosa* to these drugs (Akasaka *et al.*, 2001). We can see in Table 1 that fluoroquinolones are common targets of most of the RND efflux pumps. Overexpression of efflux pump system MexEF-OprN can induce resistance to quinolones (Kohler *et al.*, 1997, Sobel *et al.*, 2005). MexEF is repressed by MexS and positively regulated by MexT (Sobel et al., 2005). Normally elevated expression of MexEF in a *mexS* mutant requires active MexT (Maseda *et al.*, 2000). MexCD-OprJ is another efflux pump system that can extrude various antibiotics including fluoroquinolones. Expression of *mexCD-oprJ* is negatively regulated by product of gene *nfxB*, located upstream of *mexCD-oprJ*. Several kinds of *nfxB* mutants have been reported to be responsible for induction of mexCD-*oprJ* and resistance to fluoroquinolones (Jakics *et al.*, 1992, Masuda *et al.*, 1996, Poole *et al.*, 1996).

1.1.2.4 P. aeruginosa resistance to cationic antimicrobial peptides

Cationic antimicrobial peptides (CAPs) such as colistin are often used to treat infection in CF patients. The positively charged (+5) colistin targets the negatively charged lipopolysaccharide (LPS) by electrostatic interactions (Hancock & Chapple, 1999). This leads to increased permeability of the outer cellular membrane, inducing both leakage of cell contents and self-promoted uptake of colistin to the periplasm (Falagas & Kasiakou, 2005).

In *P. aeruginosa*, resistance to colistin is accomplished by adding aminoarabinose to lipid A moieties of LPS (Moskowitz *et al.*, 2004). This results in a less negative charge of outer membrane and renders the drug action. Modification of lipid A is carried out by the products of *arnBCADTEF* operon. In the presence of CAPs or low Mg^{2+} condition, two independent two-component-systems, PhoPQ and PmrAB, positively regulate the transcription of their respective operons as well as the *arnB*

operon (**Figure 3**). There is interplay of the two systems as well as cross-talks with other unknown regulators (McPhee *et al.*, 2003). Mutations in *pmrB* that cause constitutive expression of the response regulator *pmrA* and *arnBCADTEF* operon are often found in clinical isolates resistant to colistin (Moskowitz et al., 2004).

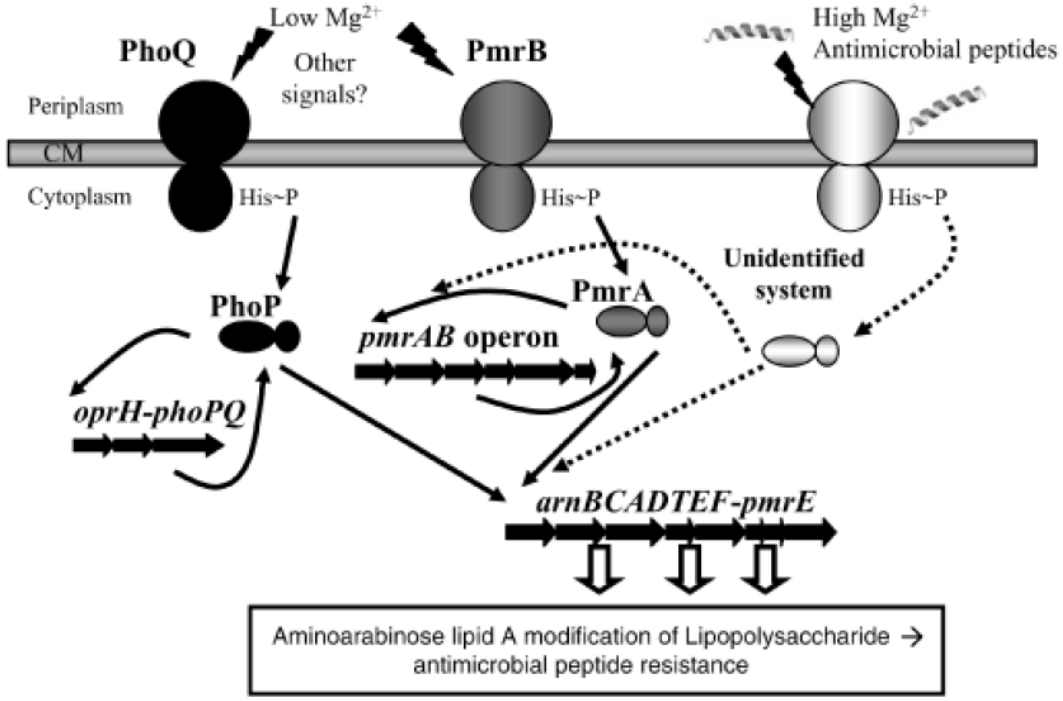


Figure 3. Regulation of *arnBCADTEF* operon and CAPs resistance (Gooderham & Hancock, 2009). PhoQ and PmrB senses Mg^{2+} limitation or CAPs and then activate their respective response regulators PhoP and PmrA by phosphorylation and positively autoregulate the transcription of their respective operons, as well as the *arnBCADTEF* operon. ArnBCADTEF pathway catalyzed the modification of lipid A, which limits the interaction of LPS and CAPs.

1.1.3 Quorum Sensing

Quorum sensing (QS) is a process of bacterial communication using secreted signal molecular called autoinducers. It is widespread in bacterial world and can occur within one species and between two species (Henke & Bassler, 2004). The concentration of extracellular autoinducer increases as the cells proliferate, and a large number of genes change their expression as a response when the autoinducer reaches a certain critical level (Henke & Bassler, 2004).

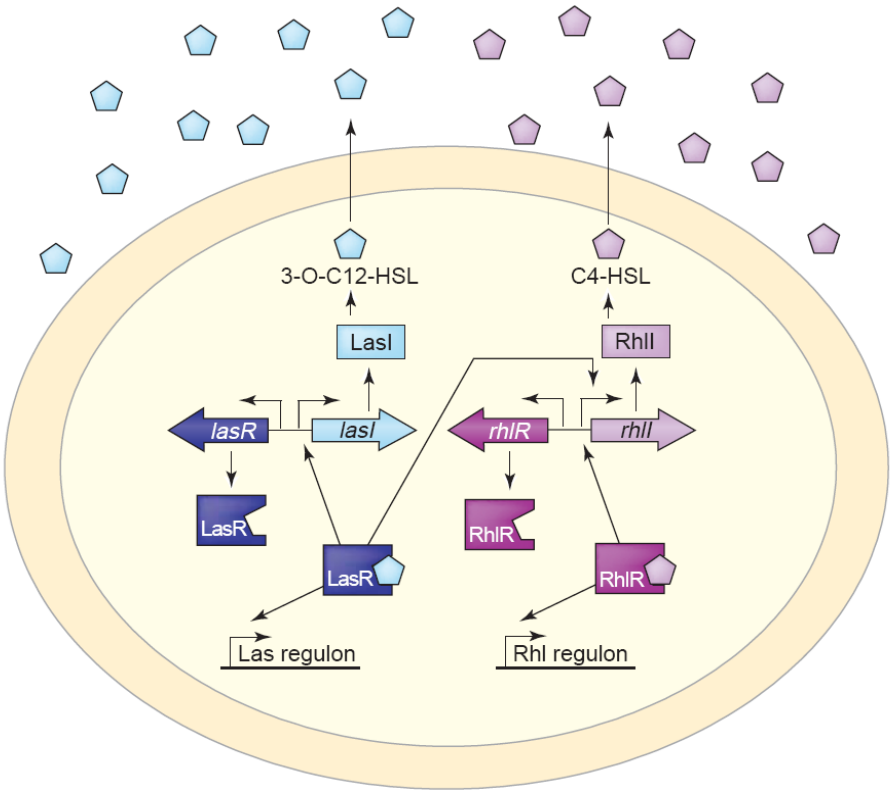


Figure 4. A simplified model of quorum-sensing in *P. aeruginosa* (Henke & Bassler, 2004). The las system (blue) and the rhl system (purple) are induced by their autoinducer 3-O-C_{12}-HSL and C4-HSL, respectively.

P. aeruginosa possesses two major quorum sensing systems, both consisting of a transcriptional regulatory protein and its cognate autoinducer molecule. For one system, the regulatory protein is LasR and its cognate autoinducer molecule, *N*-(3-oxododecanoyl)-L-homoserine lactone (3-O-C_{12}-HSL), is produced by the signal synthase LasI. When bound by its cognate molecule (3-O-C_{12}-HSL), LasR induces expression of a set of genes including *lasI*, which further increases LasI expression level and the production of 3-O-C_{12}-HSL (Seed *et al.*, 1995) (**Figure 4**). LasR also controls another QS system composed of the regulatory protein RhlR and its autoinducer, *N*-(butanoyl)-L-homoserine lactone (C_4-HSL), which is synthesized by RhlI. The RhlR is bound by the autoinducer C4-HSL and activates its target genes in a similar way. The arrays of target genes of these two systems are partially overlapping. *P. aeruginosa* also possesses a third signal 2-heptyl-3-hydroxy-4-quinolone which is referred to as *Pseudomonas* quinolone signal (PQS) (Pesci *et al.*, 1999) and it is also under the regulation of *las* and *rhl* systems.

QS plays essential roles in various biological processes of *P. aeruginosa*. There were 77 QS regulated genes agreed by three independent microarray studies (Hentzer

et al., 2003). Its function in biofilm formation and virulence factors is best studied. Biofilm is a clump of cells growing as an organised community, commonly attached to surfaces (See 1.1.4.4). It has been shown that rhamolipid and extracellular DNA are controlled by quorum sensing and they are required for biofilm development (Davey *et al.*, 2003, Allesen-Holm *et al.*, 2006). QS also regulates a set of extracellular virulence factors, which will be mentioned in **section 1.1.4.2**. In rat models of *P. aeruginosa* lung infection, QS mutant gave rise to milder immune response and tissue damage compared to the wild type especially in chronic phase (Nelson *et al.*, 2009, Wu *et al.*, 2001).

1.1.4 Virulence factors

P. aeruginosa is able to produce large variety of virulence factors (**Figure 5**) and their interactions lead to pathogenesis of *P. aeruginosa* and inflammatory responses of the host. Different roles of virulence factors are associated with different types of infections. Several major of virulence factors will be discussed here.

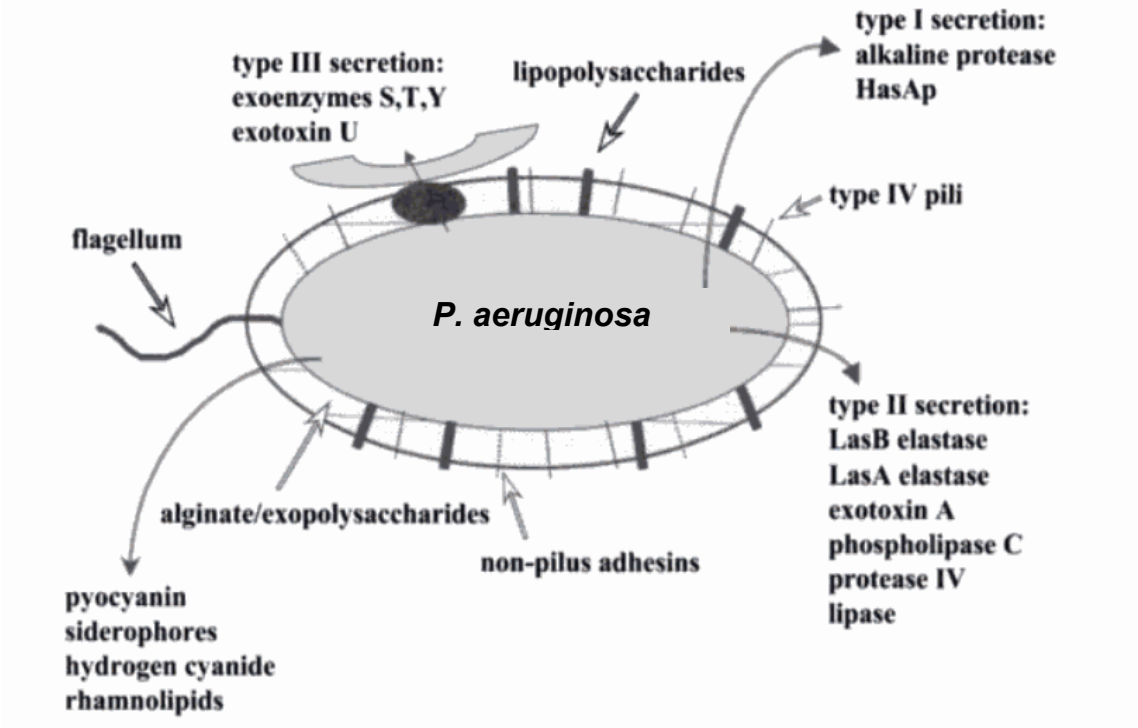


Figure 5. ***P. aeruginosa*** **virulence factors (Delden, 2004).**

1.1.4.1 Cell-associated virulence factors – Pili, Flagella, LPS

Type IV pili are dynamic filaments that are able to attach to abiotic and biotic surfaces via the tips and move the cell by extending and retracting. Type IV pili mediated

motility is called twitching. Adherence of *P. aeruginosa* to epithelial cells is mainly mediated by type IV pili (de Bentzmann *et al.*, 1996a, de Bentzmann *et al.*, 1996b). Flagellum is primarily responsible for swimming motility and chemotaxis. It has been implicated in adhesion to mucin, the main component of mucus found on the epithelium surface (Feldman *et al.*, 1998). FlhA, exporter of flagellar proteins is shown to be required in cell invasion (Fleiszig *et al.*, 2001). Flagellum is also very immunogenic, inducing both inflammation and antibody response (Mizel *et al.*, 2003, Ochi *et al.*, 1991). *P. aeruginosa* LPS is also endotoxin as in the other Gram-negative bacteria. LPS contains a lipid A region, a central core oligosaccharide region and a repeating O-linked polysaccharide portion (Delden, 2004). There are two forms of the O-polysaccharide: A-band (homopolymer) and B-band (heteropolymer), while the latter one is highly immunogenic and associated with serum resistance (Rocchetta *et al.*, 1999, Dasgupta *et al.*, 1994).

1.1.4.2 Secreted virulence factors

Secreted virulence factors in *P. aeruginosa* normally cause tissue damage, bloodstream invasion and dissemination, which are typical pathogenesis for acute infection (Delden, 2004). Extracellular virulence factors include LasA and LasB elastases, exotoxin A, lipases, phospholipase C, protease IV, alkaline protease as well as rhamnolipids, pyocyanin, siderophores, hydrogen cyanide, *etc*. Most of them are controlled by quorum sensing circuit (Delden, 2004). QS signal 3O-C_{12}-HSL itself can also induce various immune responses (Mizel *et al.*, 2003). Pyocyanin as well as pili and flagellar is regulated in an RpoN (alternative sigma factor σ54, see 1.1.6) dependent manner (Hendrickson *et al.*, 2001). Both *lasR* mutant and *rpoN* mutant have exhibited significantly reduced killing or injury in both *Caenorhabditis elegans* and mouse models (Rumbaugh *et al.*, 2000, Hendrickson et al., 2001).

1.1.4.3 Type III secretion apparatus

Type III secretion system (TTSS) is found in wide range of Gram-negative pathogens and is responsible for the invasion and cytotoxicity of *P. aeruginosa* in mammalian cells (Yahr *et al.*, 1997). TTSS injects exoproducts when contacting with target cells to elicit their toxicity at the infection site. The exoproteins, including exoenzyme S, exoenzyme T, exotoxin U and exoenzyme Y, are translocated and injected into host cells through complex machinery (Yahr et al., 1997). The genes encoding this machinery are clustered together in a locus termed exoenzyme S regulon in *P. aeruginosa* genome and the expression of these genes is controlled by the transcriptional regulator ExsA (Frank, 1997).

1.1.4.4 Biofilm

Biofilm is matrix-enclosed microbial accretions that adhere to biological or non-biological surfaces (Hall-Stoodley et al., 2004). The extracellular polymeric substance (EPS) matrix is usually composed of polysaccharides and DNA. Figure 6 illustrates biofilms growing in vivo and in lab settings. Biofilm formation is considered as a common growth mode of microorganisms in the environments. More importantly, it is associated with many bacterial infections such as *Staphylococcus epidermidis* colonization on implanted medical devices. In vitro studies revealed several important steps of biofilm development: initial attachment, microcolony formation, maturation and detachment, in which flagella, typeIV pili, QS, *etc*. are involved (Costerton *et al.*, 1999). *P. aeruginosa* is an excellent biofilm former and the formation of biofilm has been considered as one of the key virulence factors in *P. aeruginosa* chronic infection (**Figure 6**).

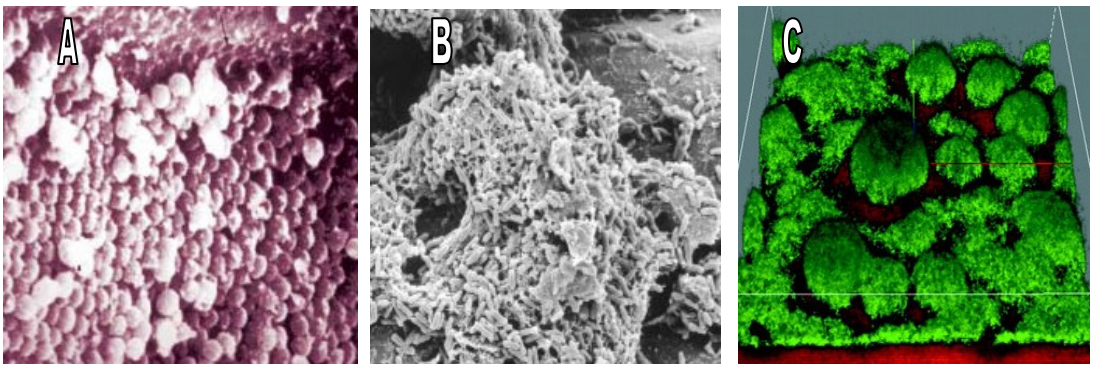


Figure 6. Biofilm in different environments. A) Scanning electron micrograph of *S. aurus* biofilm that developed on the distal tip of a cardiac pacemaker lead in a patient (Hall-Stoodley *et al.*, 2004). B) Scanning electron micrographs of a *P. aeruginosa* biofilm on the surface of a granite pebble with extracellular polymeric matrix shown (Whiteley *et al.*, 2001). C) Confocal micrograph of GFP tagged *P. aeruginosa* biofilm growing on a glass surface (Haagensen *et al.*, 2007). Red: dead cells after colistin treatment.

Biofilm associated infection is a big challenge in medical therapies (del Pozo & Patel, 2007). Figure 7 demonstrates a model explaining why forming biofilms can help bacteria persist better in the host. First of all, biofilm is more resistant to antibiotics than planktonic growing cells through multiple machanisms (del Pozo & Patel, 2007). Second, biofilms exhibit resistance to host phagocytic defences. For example, polymorohic neutrophils (PMNs) can not penetrate *P. aeruginosa* biofilm and the oxidative potential is reduced inside biofilms (Jesaitis *et al.*, 2003). Phagocytic enzymes damage single bacterial cells and host tissues. Third, antibodies also fail to

penetrate EPS matrix and mediate opsonic killing of biofilm cells (Cerca *et al.*, 2006, De Beer *et al.*, 1997).

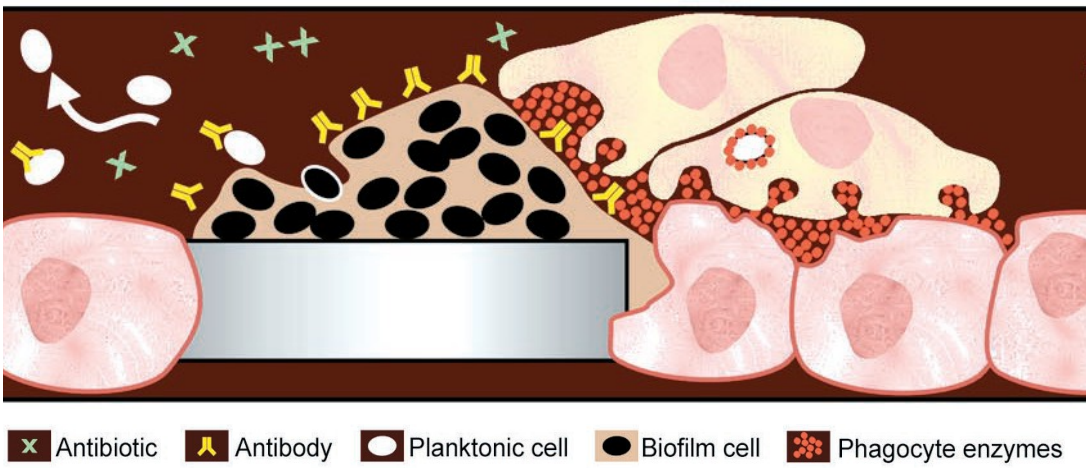


Figure 7. Model of biofilm protection against antibiotics and immune attack (Costerton et al., 1999). Compared to planktonic cells, biofilm cells are better protected from antibodies and PMNs due to the penetration problem. They are also more resistant to antibiotics.

Currently, studies of biofilm in vitro are mainly carried out in surface-associated devices with static or chemostat growth. However, to what extend these experimental settings can represent the in vivo situations is not known. The concept of biofilm has now expanded and cell aggregates that do not attach to any surfaces are also included since it is often found in vivo. For example, in a failed elbow arthroplasty case, clumps of *S. aureus* cells were mainly found in the joint fluid (Stoodley *et al.*, 2008). We should be careful when extrapolate in vitro observations into in vivo studies. The life styles of bacteria can be incredibly different depending on specific diseases even though they are all called biofilms.

1.1.5 Mucoid phenotype conversion and regulation

Mucoid phenotype of *P. aeruginosa* is often seen in CF isolates but rarely in environmental isolates. It was regarded as a major hallmark of transition from intermittent colonization to a steady persistence (Pedersen *et al.*, 1992, Govan & Deretic, 1996). Mucoidy is due to the massive amount of alginate produced from *P. aeruginosa* strains and slimy colonies are formed on agar plates (**Figure 8**). Alginate has been considered as one of the major virulence factors in CF infection. Emergence of the mucoid phenotype is associated with significantly increased rate in the decline in CF lung function (Henry *et al.*, 1992). The thick alginate layer might provide physical and chemical barrier to the bacterium to resist phagocytosis from macrophages (Oliver & Weir, 1985).

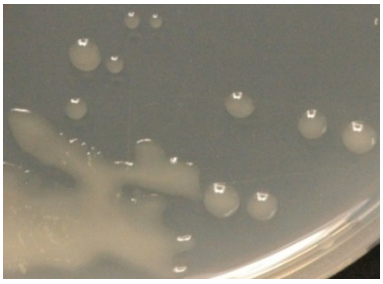
A  B 

Figure 8. Colonies of A) non-mucoid, and B) mucoid *P. aeruginosa* isolated from CF patients grown for two days on Luria–Bertani (LB) plates at 37 °C (this work).

1.1.5.1 Alginate synthesis

Alginate is a negatively charged linear copolymer of partially O-acetylated β-1,4-linked D-mannuronic acid and its C5 epimer, α-L-guluronic acid (Linker & Jones, 1966). The alginate synthesis pathway is illustrated in Figure 9. The alginates precursor guanosine diphosphate (GDP) mannuronic acid is synthesized from fructose 6-phosphate catalized by AlgA, AlgC and AlgD. This precursor is then polymized and transported across the inner membrance by a hypothized combination of *alg8* and *alg44* products (Ramsey & Wozniak, 2005). Some of the mannuronate residues are epimerized to guluronate residures by AlgG. AlgG, AlgK and AlgX might form a scaffold that protects the growing alginate polymer from being degraded by alginate lyase (AlgL) (Ramsey & Wozniak, 2005, Jain & Ohman, 1998, Robles-Price *et al.*, 2004). After epimerization, some of the mannuronate residues are acetylated at the O2 and/or O3 positions by AlgF, AlgJ and AlgI (Franklin & Ohman, 2002). AlgE is an outer membrane protein that transports alginate polymer out of the cell (Rehm & Valla, 1997).

1.1.5.2 Regulation of alginate synthesis

Transcription of the alginate biosynthesis operon (algD-algA, PA3540-PA3551) is under the control of the *algD* promoter. Alternative sigma factor AlgT (also called AlgU, σ^{22}) is the best known regulator that induces the *algD* operon transcription (**Figure 10**). The gene a*lgT* is located in an operon *algT-mucABCD*. The products of *mucA* and *mucB* bind and repress the AlgT protein. The mutation on *mucA* (or less often *mucB*) released the AlgT protein causing the conversion from nonmucoid to mucoid phenotype. Unlike other alginate genes, expression of *algC* is under the control of a two-component system response regulator AlgR, which is also activated by AlgT. AlgR also binds to *algD* promoter and cooperates with AlgT to bring a strong expression of *algD* operon (Govan & Deretic, 1996).

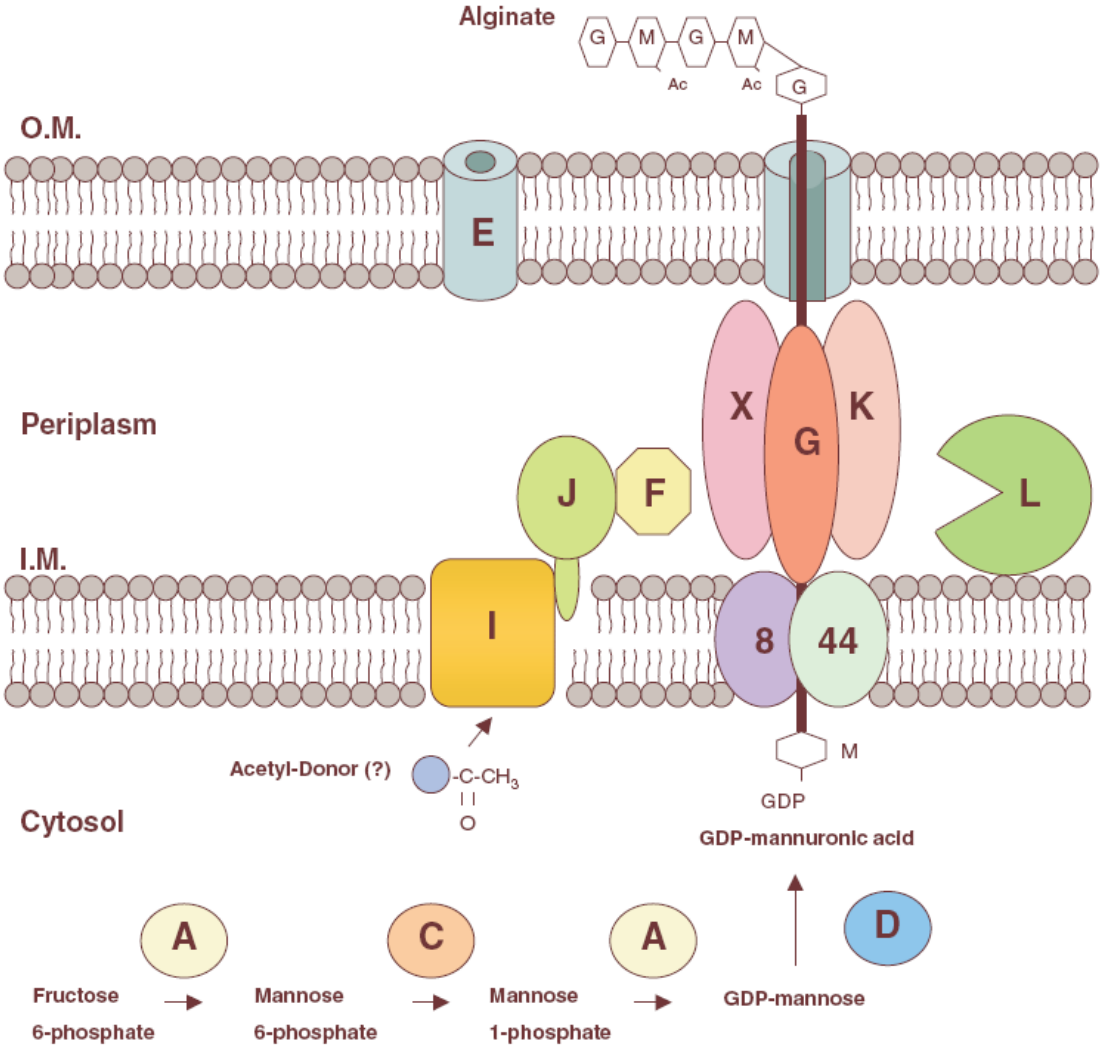


Figure 9. A model of alginate synthesis. A-X, 8 and 44 indicate AlgA, *etc.* I.M. and O.M. represent inner and outer membrane respectively. The letters G and M are guluronate or mannuronate, respectively. Ac is for acetyl group (Ramsey & Wozniak, 2005). Alginate precursor GDP-mannuronic acid is synthesized from fructose-6-phosphate, and then polymized, partially epimerized, partially acetylated and finally exported.

Besides AlgT, RpoN also plays roles in the activation of *algD* operon. Boucher *et al.* showed that RpoN is required for the mucoid phenotype in a muc23 background where there is no mutation in the *algT-mucABCD* region (Boucher *et al.*, 2000); whereas in a muc22 background where *mucA* is mutated, RpoN acts as a repressor for initiation of *algD* transcription by AlgT. The muc22 strain does not produce significant amount of alginate in a nitrogen rich medium, but it is mucoid growing on alternative nitrogen sources, such as nitrate (Deretic *et al.*, 1990). Boucher *et al.* showed that the repression is caused by antagonism of the two sigma factors RpoN and AlgT, due to the overlap of AlgT and RpoN promoters of *algD* gene. In nitrogen rich

condition, RpoN binds to *algD* promoter and inhibits its transcription regulated by AlgT; in nitrogen limited condition, RpoN is engaged in nitrogen response (see **Section 1.1.6.2**), therefore the repression is relieved and AlgT directed transcription initiates (Boucher et al., 2000).

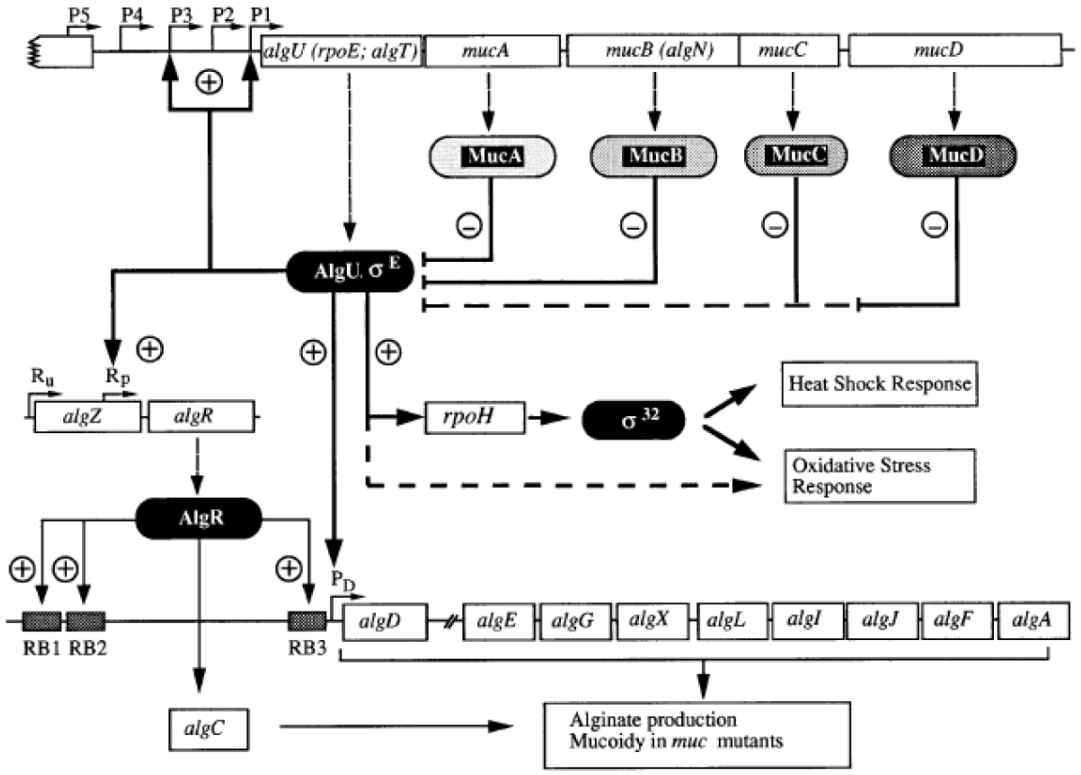


Figure 10. A classic model of alginate synthesis regulation (Govan & Deretic, 1996). The *algD* operon is positive regulated by AlgT, which is repressed by MucA (as well as MucB,C,D). AlgT induction could also lead to other effects such as oxidative stress responses.

Another regulator AlgB (belonging to a subclass of NtrC family proteins which are usually RpoN activators) is also required in alginate production. Like AlgR, AlgB controls alginate levels by activating transcription of the *algD* operon and this process does not require phosphorylation even though it is a two-component response regulator (Feldman et al., 1998). Leech *et al.* showed that AlgB binds directly to *algD* promoter and stimulates transcription from the AlgT-dependent promoter, instead of RpoN promoter (Leech *et al.*, 2008).

Damron *et al*. proposed another model which involved not only AlgB or RpoN but also the cognate sensor histidine kinase of AlgB – KinB (Damron *et al.*, 2009). In this model, it is not the *mucA* mutation but the AlgW-dependent MucA protolysis that

plays an essential role. AlgW is a homolog of DegS, a serine protease activated in response to unfolded outer membrane proteins in *E. coli* (Damron et al., 2009). KinB negatively regulates the alginate production. In a *kinB* mutant, AlgB and RpoN control the expression of factors which can activate the AlgW protease to release the repression of AlgT by MucA through proteolytix degradation and activate AlgT (Damron et al., 2009).

Additionally, the alginate and motility regulator Z (AmrZ) was shown to activate *algD* operon and AlgT is required for its activity (Baynham *et al.*, 1999). The promoter region of *algD* and binding sites of different regulators are shown in Figure 11, in which we can see the complexity of the regulation of *algD* operon.

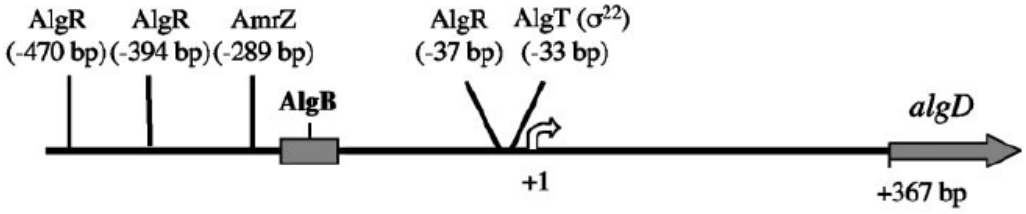


Figure 11. Binding sites of different regulators in *algD* promoter region (Baynham et al., 1999).

1.1.5.3 Pleiotropic effects of the algT activation

Due to the fact that AlgT is a sigma factor, alginate synthesis is only a subset of downstream effects of *algT* activation. Comparison of global transcriptome between a non-mucoid CF isolates and its isogenic *mucA*$^-$ strain revealed that over 600 genes had significantly different expressions ($p<0.05$, fold change>2), including many genes that encode virulence factors, such as phenazine synthesis, rhamnolopid synthesis, Type III secretion system (TTSS), flagella biogenesis, and pili assembly, *etc*. (Rau *et al.*, 2010). In accordance with these findings, it has been reported also that AlgT is a negative regulator of flagella synthesis (Garrett *et al.*, 1999). Wu *et al.* showed TTSS is repressed in *mucA* mutant mediated by induction of AlgT and AlgR (Wu *et al.*, 2004). To further clarify that the pleiotropic effects of *mucA* mutation are not the indirect consequences of over-production of alginate, Rau *et al.* also constructed an *algD* mutant in the *mucA*$^-$ background. The strain *mucA*$^-$*algD*$^-$ showed a pronounced similarity in expression profile compared to *mucA*$^-$, apart from genes directly involved in alginate synthesis (Rau et al., 2010). Phenotypically, both *mucA*$^-$ and *mucA*$^-$*algD*$^-$ strains showed dramatic decline in motility, rhamnolipid secretion, C_4-HSL production and killing of *C. elegans* compared to their non-mucoid parent strain (Rau et al., 2010). Pleiotropic effect of *mucA* mutation in reduction of virulence factor uncovered in these investigations has

important implications on *P. aeruginosa* pathogencity in chronic infections of CF patients.

AlgT has a similarity of 79% in amino acid sequence to the heat-shock sigma factor σ^E in *E. coli*. Inactivation of *algU* decreases the *P. aeruginosa* survival rate when exposed to high temperatures and diminishes its resistance to superoxide-generating redox cycling compounds (Deretic *et al.*, 1994, Yu *et al.*, 1995, Govan & Deretic, 1996). Furthermore, expression of *algU* or mucoid conversion can be induced by environmental stresses such as heat shock (Yu et al., 1995) or hydrogen peroxide (Mathee *et al.*, 1999). Another sigma factor RpoH (σ^{32}) involved in heat-shock response is dependent on AlgT as well (**Figure 10**).

In conclusion, pleiotropic effects of *algT* activation in pathogenesis are mainly to reduce the virulence factors and enhance the resistance to oxygen radicals released from the immune system. In a study of CF isolates from 115 Scandinavian patients examing *mucA* and *algT* gene sequences, only 30% of them reverted due to *algT* mutation even though many non-mucoid CF isolates also carried *mucA* mutation; whereas 83% of *in vitro* derived spontaneous non-mucoid revertants have *algT* mutation (Ciofu *et al.*, 2008). This indicates that a functional AlgT is essential for *P. aeruginosa* persistence in chronic CF infection.

1.1.6 Global regulator RpoN

We have mentioned previously about the role of σ factor RpoN regulation of *algD* operon in 1.1.5. RpoN was first reported playing essential role in nitrogen metabolism, later found to have global effects on many fundamental metabolic pathways and molecular functions. RpoN has not been investigated much for its role in *P. aeruginosa* pathogenesis. In 1994, some *rpoN* mutants were isolated from CF patients and defective *rpoN* gene was responsible for reduced swimming motility and resistance to nonopsonic phagocytosis in some of the isolates (Mahenthiralingam *et al.*, 1994). RpoN mutants were also found in chronically infected patients in later CF studies (Smith *et al.*, 2006).

1.1.6.1 Control mechanism of RpoN activators

In the transcription initiation, dissolvable σ factor associates with the core enzyme $\alpha_2\beta\beta'\omega$ to form the holoenzyme of the RNA polymerase. Like *Escherichia coli*, *P. aeruginosa* has a major σ^{70} (RpoD) that controls a large number of housekeeping genes as well as several alternative σ factors controlling various genes in different conditions. RpoN, also called σ^{54}, σ^N or NtrA, is one of the alternative σ factors but

with some unique features. First of all, unlike the other σ factors, RpoN is not homologous to σ^{70} (Merrick, 1993). Second of all, in contrast to σ^{70} like factors which are sufficient to initiate transcription by binding to the core enzyme, RpoN dependent transcription requires specific activators (Kustu *et al.*, 1991). Third of all, the binding sites of those activators can be distantly (between 100 and 200 bp) upstream from the promoters that they regulate (Weiss *et al.*, 1991).

RpoN-dependent genes generally have conserved GG-N10-GC sequence located from -24 to -12 bp of the promoter region (Barrios *et al.*, 1999). Once RpoN binds to the core enzyme, it needs to be isomerised to initialize the transcription process. The RpoN activators have ATPase activities and provide energy for the isomerisation of RpoN by ATP hydrolysis.

The RpoN activators are composed of four domains, namely A, B, C and D from amino-terminal to the carboxy-terminal of the protein (Shingler, 1996). A-domain is signal reception domain. There is little homology in A-domains of RpoN activators and they can be subgrouped depending on the modulation of activation signals. B-domain is a short link (approx. 15 to 25 residues) between A-domain and the rest of the protein. C-domain (also referred to the conserved σ^{54} activation domain) is highly conserved and involved in transcriptional activation. This domain is responsible for ATP hydrolysis and interaction with RpoN (Morett & Segovia, 1993). D-domain contains a helix-turn-helix DNA binding motif, which is analogous to transcriptional activators and repressors (Shingler, 1996).

In PAO1 genome, 22 probable RpoN activators that contain the conserved σ^{54} activation domain (Pfam00158) were found using an online server 'Integrated Microbial Genomes- Expert Review (IMG/ER) system' (http://durian.jgi-psf.org/cgi-bin/er/main.cgi) (Chaney *et al.*, 2001, Studholme & Dixon, 2003). Here we describe a few which have known functions associated with important roles of RpoN.

1.1.6.2 NtrC and nitrogen metabolism

RpoN was first identified to regulate nitrogen metabolism (Hirschman *et al.*, 1985, Hunt & Magasanik, 1985). Besides nitrogen fixation microorganisms, the first step of nitrogen assimilation in most cells is to incorporate ammonia to amino acids, where glutamate and glutamine play pivotal roles. There are two pathways of ammonia assimilation (**Figure 12**). In the first pathway, glutamate is synthesized from ammonia by glutamate dehydrogenase (GDH). In the second pathway, one molecular of ammonia and glutamate are converted to one molecular of glutamine catalyzed by glutamine synthetase (GS), followed by formation of two molecules of glutamate

from one glutamine and 2 ketoglutarate, driven by the glutamate synthase (GOGAT). GDH pathway is employed in nitrogen-rich (low carbon/nitrogen ratio) environment due to the high affinity of GDH to ammonia, while the ATP-consuming GS-GOGAT pathway is more appropriate in nitrogen-limited (high carbon/nitrogen ratio, energy rich) environment since GS has a low K_m for ammonia (Helling, 1994, Helling, 1998).

Ammonia is virtually the preferred nitrogen source for all bacterial growth and provides highest growth rate than any other nitrogen sources. However bacteria often have to utilize alternative nitrogen sources such as amino acids, nucleosides and inorganic nitrogen sources (nitrite and nitrate), which is usually referred to nitrogen limited conditions. In such conditions, bacteria are induced explore maximal synthesis of GS and produce proteins that could transport and catabolise these alternative nitrogen sources. This nitrogen-limitation induced coordinated response is called nitrogen-regulated (Ntr) response (Reitzer & Schneider, 2001).

$$NH_3 + \text{2-ketoglutarate} + NADPH \xrightarrow{GDH} \text{glutamate} + NADP^+$$

$$NH_3 + \text{glutamate} + ATP \xrightarrow{GS} \text{glutamine} + ADP + P_i$$

$$\text{Glutamine} + \text{2-ketoglutarate} + NADPH \xrightarrow{GOGAT} 2\ \text{glutamate} + NADP^+$$

Figure 12. Ammonia assimilation (Merrick & Edwards, 1995). Ammonia assimilation is carried out by GDH pathway in nitrogen rich condition and GS-GOGAT pathway in nitrogen limiting condition.

In *P. aeruginosa* Ntr response is controlled by a four-protein-system through glutamine and GS (encoded by *glnA* gene): the uridylyltransferase (UTase)-uridylyl removing (UR) enzyme (encoded by *glnD*), a small trimeric protein, P_{II} (encoded by *glnK*), and a two-component regulatory system composed of the histidine protein kinase NtrB and the response regulator NtrC. In high glutamine conditions (nitrogen-rich), UR is activated which prevents uridylylation of P_{II}. Unmodified P_{II} interacts with NtrB and stimulates the dephosphorylation of NtrC. The operon *glnA-NtrBC* is only expressed in a basal level. When glutamine level is low (nitrogen-limited), P_{II} - UMP complex is formed and prevents the interaction between P_{II} and NtrB. NtrB autophosphorolates and transferes the active phosphate group to NtrC. NtrC-P activates the expression of *glnA-NtrBC* operon through RpoN (Reitzer & Schneider, 2001). A *P. aeruginosa* strain with deficient NtrC or RpoN is expected to grow defectively in low extracellular ammonia concentrations (nitrogen-limitation). There

is a second regulation system of Ntr response in *E.coli*, Nac regulon. It works through σ^{70} dependent promoters but it does not have homologues in Pseudomonas (Li & Lu, 2007).

The mechanism of transcription activation of *glnA* promoter by NtrC in *Salmonella typhimurium* is illustrated in **Figure 13**. NtrC has two binding sites at -108 and -140 for dimers (A), which are very distant from the *glnA* RpoN promoter sequence (-12 and -24). Phosphorylation and oligomerization of NtrC is required in the process (Porter *et al.*, 1993). Phosphorylated oligomer of NtrC contacts RpoN-holoenzyme by means of DNA loop formation (B). NtrC then hydrolyzes ATP and catalyzes isomerisation of RpoN to open the closed complexes. The region around the transcriptional start site denatures and transcription is activated (C).

Besides *glnA*, NtrC also regulates many other genes in *E. coli* in the similar way (Merrick & Edwards, 1995). Jones *et al.* identified at least 11 operons or genes in *Pseudomonas fluorescens* Pf-5 having RpoN and NtrC binding sites in the upstream that are predicted to be involved in nitrogen assimilation, including *glnA*, *ureE* (Urease accessory pritein UreE), narX (Nitrate/nitrite sensor protein NarX) and PFL1000 (a putative amino acid transporter) (Jones *et al.*, 2007). Those finding are in consistent with the poor performance of an *rpoN* knock-out strain utilizing nitrate, urea and many amino acids as nitrogen sources (Jones et al., 2007).

1.1.6.3 CbrB and carbon metabolism

Nishijyo *et al.* first discovered a two-component system CbrA-CbrB in *P. aeruginosa* that controls the catabolism of histdine and arginine (Nishijyo *et al.*, 2001). Li *et al*. further characterized the interplay of CbrAB and NtrBC in the control of histidine and arginine operons (Li & Lu, 2007). They found that the growth of *cbrAB* mutant on arginine or histidine as sole carbon sources was abolished, while growth on the tricarboxylic acid (TCA) cycle intermediates was sustained. An *ntrB* mutant that has a constitutive activation of NtrB in *cbrAB* mutant strain can restore the growth of arginie and histidine as carbon sources, but the capability of growing on TCA intermediates and glucose was compromised when ammonium is used as nitrogen source. This indicates that CbrAB and NtrBC form a network to control the C/N balance in *P. aeruginosa* (Li & Lu, 2007).

In *Pseudomonas*, the products from a *hut* locus are responsible for histidine uptake and degradation. This locus was well described in *Pseudomonas fluorescens* SBW25 strain (Zhang & Rainey, 2007).The homologs of *P. aeruginosa* were identified to be

coded by gene cluster PA5091 to PA5106 (Zhang & Rainey, 2007). Histidine is uptaken by a histidine transporter (encoded by *hutT*), and then degraded to glutamate, formate and ammonium by a five-step pathway catalyzed by enzymes encoded by *hutUHIG* and PA5106 (*hutF*). HutC is a local repressor of *hut* genes, but derepression of HutC is insufficient to activate the transcription of *hutU* operon (Zhang & Rainey, 2008).

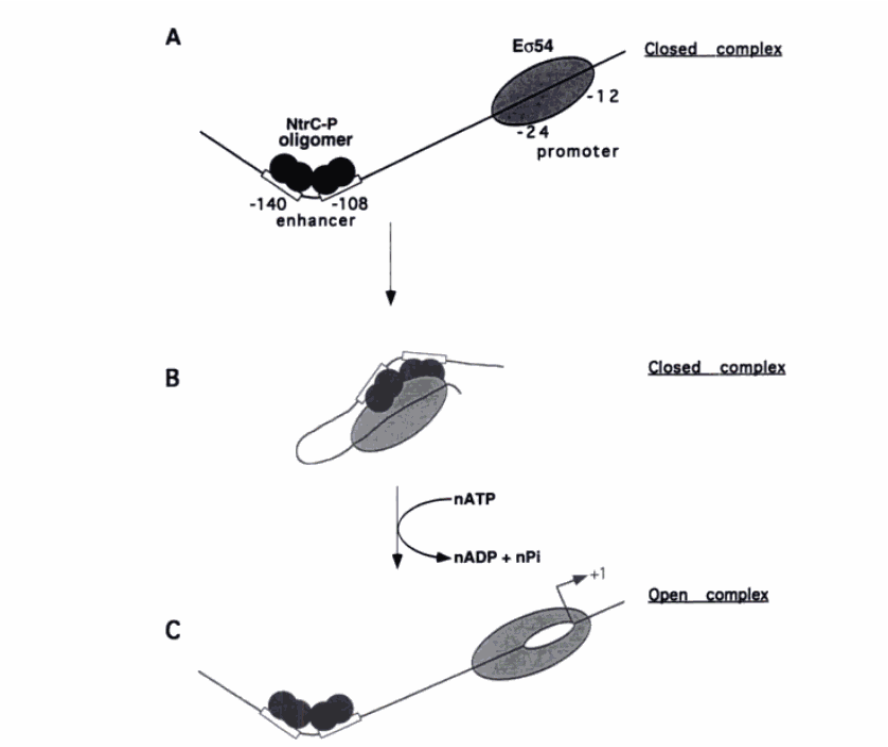


Figure 13. RpoN activation by NtrC (Porter, 1995). NtrC forms oligomer and binds to the *glnA* promoter. The NtrC oligomer then contacts RpoN and catalyzes its isomerisation by ATP hydrolysis. A localized denaturation around transcription start site occurred to initiate transcription.

Zhang *et al*. demonstrated the regulation of histidine utilization in *P. fluorescens* SBW25 (Zhang & Rainey, 2008). When histidine is the sole carbon source, transcription of *hutU* operon is initiated from a σ^{54}-type promoter and CbrB is the activator; when histidine is the sole nitrogen source, *hutU* operon is transcribed from a σ^{70}-type promoter. The exact mechanism of the latter one is not known, but neither CbrB nor NtrC is required. They also showed that CbrAB is capable of maintaining *hut* activity across a wide range of C:N ratios, while NtrC is only involved in *hut* activation in high C:N ratio (Zhang & Rainey, 2008). This regulation network is very different from other bacteria. In enteric bacteria, histidine is degraded by a four-step

pathway into glutamate and formamide. The extra step which converting formamide to formate and ammonium enables Pseudomonas to extract carbon from histidine more sufficiently, with more ammonia produced as well. Even though the accumulation of ammonium will inactivate NtrC, CbrB can still regulate the transcription of *hut* operon. This indicates the existence of CbrAB is an adaptive advantage for Pseudomonas to utilize various nutrients in the environments.

Besides histidine, CbrB also regulates the metabolism of arginine in *P. aeruginosa*. In aerobic conditions, arginine is transported into cells via an ABC transporter system encoded by the *aotJQMJ* genes and converted to glutamate by the arginine succinyltransferase (AST) pathway encoded by the *aruCFGDBE* genes (Li & Lu, 2007). The arginine-specific regulatory protein ArgR is essential for the activation of both *aot-argR* and *aruC* operons, whereas the *aotJ* promoter also depends on CbrB and RpoN (Nishijyo *et al.*, 2001).

A *cbrB* mutant of PAO1 is also unable to grow on proline as sole carbon and nitrogen sources. However, this is not caused directly by failure of transcription activation in which CbrB and RpoN are involved, but by metabolic block elsewhere (Nishijyo et al., 2001).

1.1.6.4 FleQ, FleR and flagellar synthsis

Transcriptional regulator FleQ and two component system FleSR are adjacent in the *P. aeruginosa* genome. FleQ and FleR are RpoN activators and they control the expression of most of the flagellar synthesis genes. Dasgupta *et al.* proposed a model of regulatory circuit of flagellar biogenesis in PAK strain (Dasgupta *et al.*, 2003), illustrated in **Figure 14**. FleQ is the master regulator that directly or indirectly controls the expression of the majority of flagellar gene promoters except *fliA* (Dasgupta et al., 2003). FleQ and RpoN directly regulate the transcription of structural genes *fleEFGHJ, glhA, flgG* and *fliLMNOPQRflhB*, encoding proteins forming the basal body, MS ring, P ring, motor, switch, flagellar export apparatus and the filament cap. FleQ also regulates four regulatory genes, *flhF* (determines the flagellar localization), *fleN* (crucial in maintaining a single flagellum) and *fleSR*. FleR and RpoN activate the genes that encode the basal body-rod, L ring, hook, hook-cap scaffold and hook filament junctional proteins. A *P. aeruginosa* mutant of RpoN does not express flagellin genes(Totten *et al.*, 1990).

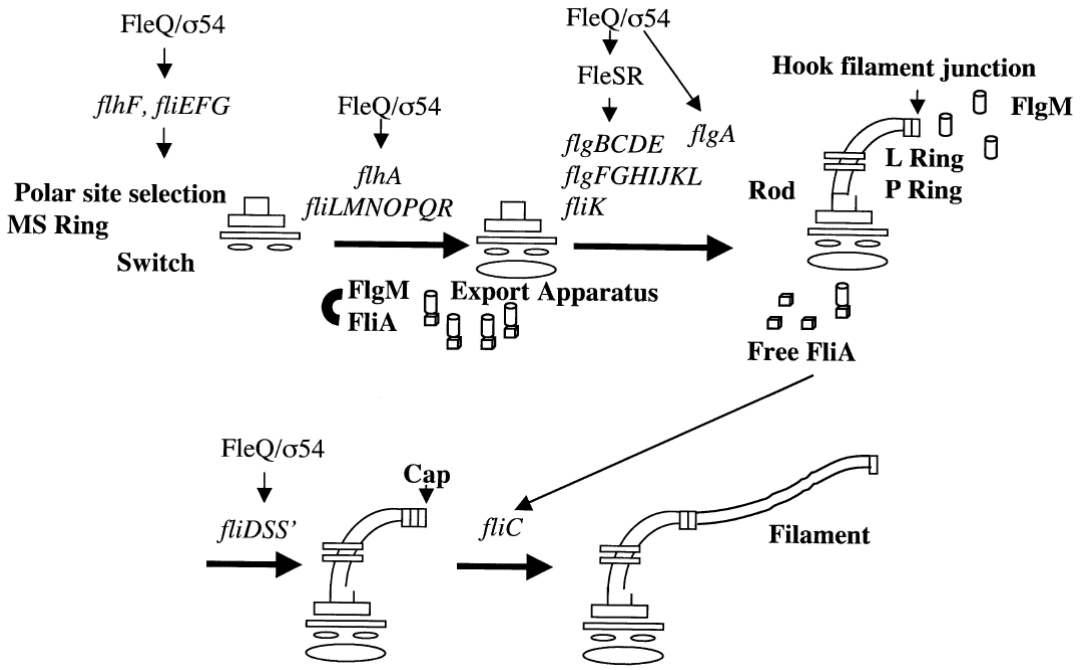


Figure 14. Flagellar synthesis and regulation (Dasgupta *et al.*, 2003). FleQ and RpoN are involved in regulation of most of the flagellum genes.

1.1.6.5 PilR and pili synthesis

Type IV pili are required for microbial adhesion on plastic surfaces and host cells, as well as movement based on pili retraction (twitching). There are many genes involved in pili assembly and regulation in *P. aeruginosa*. One major component of the type IV pili is pilin, encoded by *pilA* gene. The transcription of *pilA* is controlled by RpoN and two component system PilSR, while PilR is an RpoN activator (Hobbs *et al.*, 1993, Boyd *et al.*, 1994). An RpoN mutant is not able to assemble pili or adhere to epithelium (Ramphal *et al.*, 1991, Simpson *et al.*, 1992, Ishimoto & Lory, 1989) .

1.2 Cystic Fibrosis and pathogenesis

1.2.1 Cystic Fibrosis

Cystic Fibrosis (CF) is a life threatening genetic disease that affects about 70,000 children and adults worldwide (www.cff.org). It is most common in Caucasian populations. In 1997, the incidence is 1: 3300 in Caucasians in United States, and only 1:15300 and 1:32100 in African Americans and Asian Americans, respectively (Strausbaugh & Davis, 2007). In 2008, the median predicted age of survival is 37.4 years (www.cff.org). The cause of CF was identified in 1989 to be mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene, located in

chromosome 7 (Kerem *et al.*, 1989, Riordan *et al.*, 1989). The most common mutation in *CFTR* gene is an in frame deletion of a phenylalanine at position 508, known as ΔF508, with a frequency of 80% in Danish CF patients and 70% worldwide (Collins, 1992, Koch & Hoiby, 1993).

CFTR gene encodes AMP-dependent chloride channel which controls the passage of chloride ions in and out of the cell membrane. When a cell is stimulated to secrete, Cl^- channels open allowing Cl^- to pass through the membrane down its electrochemical gradient. Na^+ ions follow the Cl^- ions through a paracellular pathway, and water follows the salt due to the osmotic gradient (Lyczak *et al.*, 2002). CFTR is mainly expressed in epithelial cells in respiratory, gastrointestinal, hepatobiliary and reproductive systems. Thus, CF is a multisystem disease characterized by GI and nutritional abnormalities, salt loss syndromes and male urogenital abnormalities, but the major clinical problem for CF patients is chronic pulmonary disease (Campodonico *et al.*, 2008). In the lung, CFTR is distributed both in the conducting zone (consisting bronchioles) and respiratory zone (consisting alveolar)(Engelhardt *et al.*, 1994) (**Figure 16**). Mutated CFTR results in unbalanced ion composition of epithelial cells secretion and dehydrated thick mucus accumulation (Sanders *et al.*, 2000). Impaired pulmonary defense leads to colonization of pathogenic microbes in the lung, which causes inflammation, scaring of lung tissues, insufficient air exchange and gradual reduction of lung function. The detailed mechanism of pathogen colonization and infection progression is discussed in **section 1.2.3**.

1.2.2 Pathogenesis of CF lung infection

Figure 15 shows the prevalence of the major bacterial pathogens in relation to age in CF patients, including *P. aeruginosa*, *Staphylococcus aureus*, Methicillin-resistant *Staphylococcus aureus, Haemophilus influenzae, Burkholderia cepacia* complex, and *Stenotrophomonas maltophilia. S. aureus* and *H. influenzae* are the most common bacteria species found in infants and CF children. Their role in the pathogenesis in CF patients is still not clear, but it has been proposed that they might damage the epithelium surface, leading to better attachment for *P. aeruginosa* (Campodonico *et al.*, 2008, Lyczak *et al.*, 2002).

P. aeruginosa is considered as the most crucial pathogen in CF patients. Up to 80% of the adult patients carry *P. aeruginosa* (**Figure 15**). The presence of *P. aeruginosa* in the respiratory tract and the inflammatory responses it leads to are associated with increased rate of deterioration of lung function and risk of death (Campodonico et al.,

2008, Li *et al.*, 2005). A CF sputum sample can have up to 10^9 *P. aeruginosa* cells per ml (Hoiby, 1998). It is not clear why *P. aeruginosa* in general can proliferate better than other bacteria in CF lungs. One explanation is that wild type CFTR can bind to *P. aeruginosa* specifically and mediate an inflammatory response to eliminate infecting organisms, but this elimination process is impaired in CF patients (Campodonico *et al.*, 2008). There are also evidences that *P. aeruginosa* can repress the growth of *Staphylococcus* spp. in vitro or in vivo (Mashburn *et al.*, 2005, Qin *et al.*, 2009).

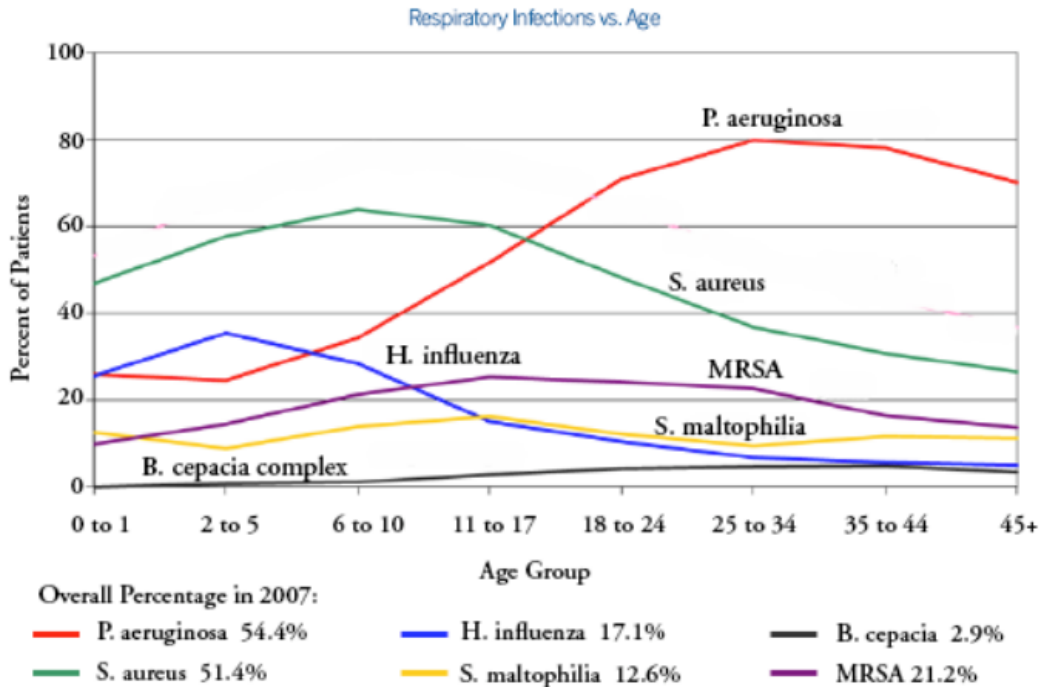


Figure 15. Prevalence of the major bacterial pathogens in CF patients in relation to age (Goldberg, 2010). *P. aeruginosa* is the most frequent pathogen found in adult patients.

Recent studies using non-culture detection methods have identified new bacteria species that could be potential pathogens, including some anaerobes (Harris *et al.*, 2007). Besides bacteria, eukaryotic microbes like Aspergillus spp are often isolated from CF sputum. Many different viruses have also been detected in CF patients, such as respiratory syncytial virus and influenzae A and B (van Ewijk *et al.*, 2005). Bacterial phages also draw attention to researchers to understand the bacteria physiology in CF lungs. It is now generally appreciated that the CF airway is a complex and diverse ecosystem and investigation of the interaction of different microorganisms in the context of lung environment will contribute significantly to our knowledge of infection in CF patients.

1.2.3 CF lung environments – indications of selective stresses for bacteria adaptation

The human lungs contain the conductive zone and the respiratory zone (**Figure 16**). The conductive zone, which is 5% of the total lung volume (about 150 ml), includes the trachea, bronchi, and terminal bronchioles (Hoiby, 2006). The epithelium of this zone contains mixed ciliated and non-ciliated cells in a firm layer. Conductive zone

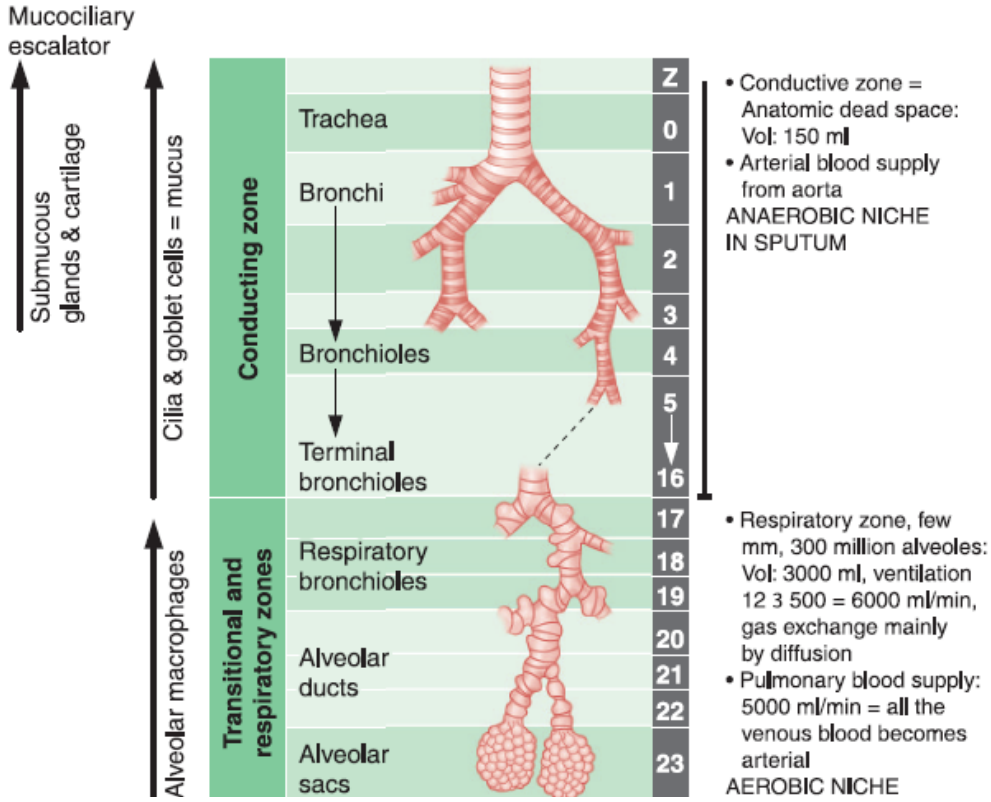


Figure 16. Conductive and respiratory zone of the lungs (Hoiby, 2006)

produces mucus, which is a thin layer of 1-2% aqueous solution of mucins, enzymes, bacteria cells and products, antibacterial agents, cell debris from both bacteria and immune cells such as DNA and other mediators and proteins. Mucins are high molecular weight glycoproteins secreted from epithelial goblet cells and mucosal cells of submucosal glands. In healthy individuals, mucins are the main contributors of moderate viscosity of mucus. Inhaled particles or bacteria are trapped in mucus and exported from the airway by beating of the cilia (forming sputum), a process termed mucociliary clearance, which is the first line of pulmonary defense against microbial pathogens. CF patients on the other hand, have much more viscous mucus due to dehydration and more importantly, massive amount of DNA released from the cell debris. Cilia can not beat sufficiently to remove the mucus layer and impaired

pulmonary defence leaves a chance for microbial colonization. DNase is often used in clinic to reduce the viscosity of mucus for better air flow in the conductive zone.

Figure 17 illustrates that *P. aeruginosa* cells form clumps embedded in the thick mucus intraluminally (not attached to bronchial wall or epithelial tissue) (Bjarnsholt *et al.*, 2009, Worlitzsch *et al.*, 2002). Even though there is an arterial blood supply from the aorta, the conductive zone is generally regarded as anaerobic or microaerobic for bacteria growth in the thick mucus layer (Worlitzsch et al., 2002). Nitrate which is an electron receptor for *P. aeruginosa* anerobic growth was found also in sputum samples with sufficient concentration to support the growth (Palmer *et al.*, 2007).

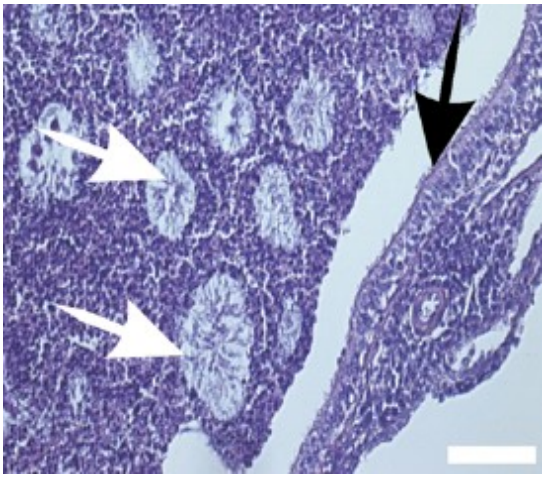


Figure 17. Thin section of an obstructed CF bronchus, stained with hematoxalin/eosin (Worlitzsch et al., 2002). Black arrow indicates the absence of *P. aeruginosa* on epithelial surface and white arrows show the presence of *P. aeruginosa* macrocolonies within intraluminal material. The blue gap is an artifact due to fixation. Scale bar: 100 μm.

The nutrient supply in the mucus is mainly from cell lysate of immune cells, epithelial cells, lung/blood tissues or bacteria and considered to be rich. A study analyzing the components of CF sputum samples revealed that amino acids are abundant in the mucus (Palmer et al., 2007). It has been shown recently that alanine is a preferred carbon source, but the study was performed using a lab strain instead of adapted CF isolates (Boulette *et al.*, 2009). A microarray study directly on sputum samples demonstrated un-regulation of genes related to degredation of phosphatidylcholine (lung surface liquid molecues), indicating lung surface liquid might serve as another nutrient (Son *et al.*, 2007).

The respiratory zone which accounts for 95% of the lung volume (around 3000ml) contains respiratory bronchioles, alveolar ducts and sacs, but no cilia or submucosal

glands (Hoiby, 2006). The respiratory zone has venous blood passing through the capillaries of the aveoles and is considered as an oxygen rich niche for bacteria growth. But if the mucus blocks the air flow, the oxygen concentration drops in the respiratory zone. Chronic infection of bacteria in respiratory zone is usually associated with more severe lung destruction than infection in only mucus in conductive zone. Even in patients who have no persistence of *P. aeruginosa* within the respiratory zone, alveoli can still be damaged due the intensive immune response against *P. aeruginosa* attack.

When nebulized tobramycin or colistin is inhaled by the patients, the concentrations of the drugs can be very high in the conductive zone, but low in the respiratory zone. However, if the intravenous course is administrated, concentrations of antibiotics are high in respiratory tissues but low in sputum (Hoiby, 2006). A microscopy investigation of lung tissue specimen and sputum samples from the Copenhagen CF centre showed that inhaled antibiotics can reduce but not eradicate the bacteria in the conductive zone; intravenous antibiotics administration on the other hand efficiently protects the respiratory zone from massive bacteria colonization (Bjarnsholt *et al*., 2009). Fixed lung material from patients who died before intensive treatment was introduced in the 1970s was preserved and examined as well. The results showed that both conductive and respiratory zone (i.e., alveolar ducts and sacs) have abundant bacteria aggregates (Bjarnsholt *et al*., 2009). This indicates as least in the Copenhagen CF centre, where there is also centralized control, intensive chemotherapies and special strain dynamics (see **section 1.2.4** and **section 1.3.3**), mucus layer is the main reservoir for *P. aeruginosa* colonization and adaptation.

Apart from disabled mucociliary clearance, CF patients have functional immune systems. Different immune defenses recruited include polymorphonuclear leukocytes (PMN), alveolar macrophages, and antibodies. When PMNs phagocytose bacteria, highly reactive oxygen species (ROS) are produced, which kill bacteria or induce mutations, as well as damage surrounding host tissues (Hoiby, 2006). PMNs can be rich in both conductive zone and respiratory zone.

In conclusion, there might be different niches in the lung for bacteria colonization in CF lungs, but mucus layer in the conductive zone is one the most important ones where most of the CF studies focused on. Nutrients, oxygen concentration, antibiotics concentrations, immune defences, *etc.* could be important environmental factors that drive the adaptation of bacteria in the long term colonization in CF lungs. Some of these factors like nutrients might be relatively stable over time and similar between

individuals; while others like antibiotics concentrations can alter all the time and differ from different patients or clinics. These lung environmental clues must be taken into consideration when studying *P. aeruginosa* persistence in CF lungs.

A lot of efforts have been put to make animal models that could mimic the human CF lung condition especially for chronic infection. CFTR knock-out mice and pigs have been generated to study CF. Due to the difference in genetic background, disruption of *CFTR* gene in pigs develop abnormalities in various organs that are much more similar to humans compared to mice, including lung pathology (Rogers *et al.*, 2008a, Rogers *et al.*, 2008b). But it is more difficult to perform studies with pigs than mice for obvious reasons. It is still remains to be investigated whether CFTR-/- pigs develop spontaneous lung infections and follow the same dynamic as the infection progresses as in humans.

1.2.4 Copenhagen CF centre: strain collection, treatment and definition of infection phases

Copenhagen CF centre was established in 1968 for centralized control and treatment of CF patients in Rigshospitalet (Copenhagen University Hospital) (Nielsen *et al.*, 1988). The patients were examined on a monthly basis for lung function, antibodies, microbiology of lower respiratory tract secretion, *etc*. The patients, since the year 1976, were administrated intravenous courses that last for 14 days every three months with selected combinations of antibiotics (normally one from β–lactams and one from aminoglycosides). After the year 1984, the patients also started to take daily treatment with ciprofloxacin combined with either inhaled tobramycin or colistin (personal communication with Dr. Helle Johansen).

Pulse Field Gel Electrophoresis (PFGE) was used as a main typing method to study the epidemiology of chronic *P. aeruginosa* infection. Epidemic spread of multi-resistant strains in the Copenhagen CF centre was reported and patients separation was introduced to prevent transmission (Pedersen *et al.*, 1986). Since the year 1981, patients with positive bacterial colonization were separated from those without. Afterward, they were further segregated into several cohorts: intermittent infected patients, chronic infected patients, patients without *P. aeruginosa, etc.* This leads to a special strain dynamics in the Copenhagen CF centre, which is described in **section 1.3.3**.

Valuable *P. aeruginosa* isolates and documentation about the patients and in the Copenhagen CF centre were available since 1973. Normally one pair of mucoid and

non-mucoid colonies from one sample were stored, but sometimes more colonies were stored if different morphologies appeared. The storage of *P. aeruginosa* isolates was not done routinely but irregularly until 2005, when we started to freeze all the samples delivered to the clinic. Meanwhile the whole sputum samples have been stored instead of single isolates.

P. aeruginosa colonization is used as an essential indicator of infection stages since it is the most prevalent pathogen in CF patients. Chronic *P. aeruginosa* lung infection is defined as persistent presence in the sputum culture of *P. aeruginosa* for at least 6 consecutive months, or less when combined with the presence of two or more *P. aeruginosa* precipitins (Hoiby *et al.*, 2005). This definition is proposed in the Copenhagen CF centre but used in other clinics as well. A period of intermittent colonization precedes the chronic lung infection and starts from the first positive *P. aeruginosa* cultivation from sputum (**Figure 18**). Intermittent colonization could last

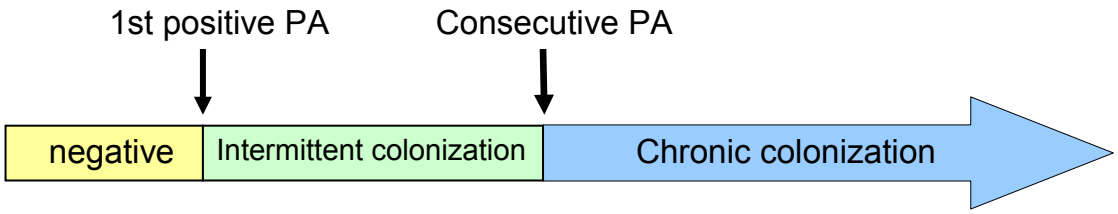


Figure 18. Infection stages of CF lungs. PA: *P. aeruginosa*. Intermittent colonization can be eradicated and patients can stay PA negative for a period of months or even years. This process repeats until chronic colonization is established.

from several months to a couple of years and is typically characterized by repeated cycles of short colonization and eradication and each cycle is most often caused by different clones. This definition is stated from clinical point of view. How different infection phases correlate with detailed patterns of bacterial adaptation still needs to be determined.

Since chronic colonization is impossible to eradicate, treatment of CF patients in their early age became an important window to delay the chronic infection and preserve the lung function. Early aggressive therapy using ciprofloxacin, colistin and tobramycin has successfully improved the survival of patients without serious side effects (Hoiby et al., 2005). Recently surgeries in sinus have been introduced to the Copenhagen CF centre to remove the visible colonized tissue/pus in the paranasal sinuses of CF children who have symptoms of sinus infection (Johansen *et al.* unpublished data). Since bacteria in the sinuses may have less risk of meeting

antibiotics and they can also be transported to the deep lower respiratory tract, one hypothesis is that they adapt prechronically in the sinuses and spread afterwards into the lung. Several investigations already support this hypothesis (Mainz *et al.*, 2009, Hansen *et al.*, unpublished data) and sinuses could be an important niche for *P. aeruginosa* adaptation. Sinus surgeries in the early stage of infection could be another effective way of preventing or delaying the chronic infection.

In summary, there are four major features in the Copenhagen CF centre that are important in order to understand the later conclusions of *P. aeruginosa* infection. First, Copenhagen CF centre executes an intensive antibiotic chemotherapy (including intravenous courses) from early ages of the CF patients. This might induce more resistance of *P. aeruginosa* to antibiotics but keeps the lung functional for a longer time. This intensive treatment is not applied in many other CF clinics, where multi-resistance is concerned more problematic. Second, CF patients in the same cohort have chances of strain transmission. Third, there are two phases of CF infection – intermittent phase and chronic phase. Finally, a unique collection of *P. aeruginosa* isolates and documents since 1970s allows us to study the epidemiology and adaptation dynamics.

1.3 Motivation and strategy of the project

1.3.1 Previous ideas and models about *P. aeruginosa* persistence

Previous investigations of *P. aeruginosa* and CF infection were mainly focused on how virulence factors can cause inflammation and tissue damage, how transition to the mucoid phenotype provides advantages for the persistence, if biofilm growth mode determines the colonization and persistence, *etc*. More and more evidences show that classical virulence factors such as motility, QS, proteases, type III secretion, LPS, *etc*. are phenotypes that chronic CF isolates tend to loose (Lee *et al.*, 2005, Hanna *et al.*, 2000, Hancock *et al.*, 1983, Luzar & Montie, 1985). Virulence factors are mainly required in acute infection, but seemingly not in chronic infections (Nguyen & Singh, 2006). Converting to mucoid phenotype is occurring with high frequency and has been considered as a hallmark of chronic stage of the infection. However, there are patients who do not carry mucoid strains constantly and the key factor for these non-mucoid strains to be successful colonizers is not known. It is also appealing to find out if mucoid and non-mucoid strains have some common pathways to adapt or they simply occupy different niches.

For a long time, biofilm has been accepted as a symbol of chronic infection (Furukawa *et al.*, 2006). Biofilm formation can lead to multi resistance to antibiotics (Hendrickson *et al*., 2001) and some toxins required for acute infection such as TTSS is actually incompatible with biofilm formation (Kuchma *et al.*, 2005). However, a study on the biofilm formation ability of longitudinal CF isolates showed that non-mucoid *P. aeruginosa* isolates exhibited abnormality in their biofilm development and generally reduced capacity of *in vitro* biofilm formation as the infection progressed (Lee et al., 2005). Another study found that the *lasR* gene, a key player in *P. aeruginosa* biofilm development is frequently mutated in CF isolates (Smith et al., 2006). All these results suggest that even though biofilm might exist in the lung, it is not structured exactly the same as what we see in *in vitro* settings. If so, what is the real life style of these bugs in the CF infection? Is switching growth mode enough to explain the complexity of *P. aeruginosa* infection in CF?

Several studies seeking CF specific features in genome contents have finished using whole-genome sequencing or DNA microarrays (oligonucleotide array). There were both gaining and loss of specific segments in different strains, but no shared patterns were found in chosen CF isolates (Mathee *et al.*, 2008, Ernst *et al.*, 2003).

Although mutations in *mucA* and B-band O-antigen have been reported in many CF studies (Hancock et al., 1983, Luzar & Montie, 1985), the concept of 'genetic adaptation' was first brought to the study of *P. aeruginosa* in CF infection only in 2006. Smith *et al*. sequenced two genomes isolated from one patient at 6 months and 96 months of infection (Smith *et al.*, 2006). 68 mutations were found including a DNA mismatch repair gene *mutS*. They have also cross-checked 91 isolates from 29 other CF patients. The most common mutational targets are *mexZ*, a negative regulator of multi-drug efflux pump, and *lasR*, primary regulator of quorum sensing and many virulence-related genes. By tracing *mucA* mutation in intermediate isolates they discovered multiple lineages coexisting in the early stage of the infection. The major and most striking finding in this paper is that QS and virulence factors are selected against in the CF adaptation of *P. aeruginosa*.

The outstanding work by Smith *et al*. provided important information about the adaptation and diversity of *P. aeruginosa* infection in CF patients, but further questions were also laid out in front of us. Since this study mainly covered the first 7.5 years of the infection, where does the adaptation go as the infection progresses? Is there an 'optimum' of the evolution? If this is correct, is this 'evolutionary optimum' common in different patients or clinics? What is the impact of each mutation on the

adaptation? Does the evolution always proceed with the same rate? Since we do not know the epidemiology of the strains in this study, are there strain transmissions that could interfere with our analysis of the evolution? We hope to be able to answer some of these questions in our study.

1.3.2 Molecular epidemiology and dynamics of *P. aeruginosa* isolates from the Copenhagen CF centre

The ground work of this thesis was done by Jelsbak *et al* who investigated the epidemiology of *P. aeruginosa* populations in the Copenhagen CF centre using single-nucleotide polymorphism (SNP) typing tool – AT biochip (Clondiag Chip Technologies, Germany). A large amount of isolates from both chronic and intermittent patients were studied.

High genotype diversity was found in intermittently colonized patients and recently diagnosed chronically infected patients. Most of those patients acquire unique clones from the environment. Early aggressive chemotherapy can temporarily remove these bacteria but re-colonization by identical or different clones occurred later (Jelsbak *et al.*, 2007).

In contrast, there is very low genotype diversity among CF patients with long-term chronic infection. The distinct and dominant clones (r and b) were identified and they have been present and transmitting among different patients for about thirty years (**Figure 19**). The transmission is most likely because these patients with chronic infection were centralized for treatment as a cohort in the Copenhagen CF clinic.

The two dominant clones have evolved into highly successful colonizers of CF patients and they are capable of out-competing other strains including environmental strains. The study also showed that virulence factors or conversion to mucoid phenotype play minor roles in the persistence and transmission of these two dominant clones (Jelsbak *et al.*, 2007). The pathways that are really involved in the development of persistence of successful *P. aeruginosa* colonizers still need to be identified. The parallelism or divergence of the same genotype in different patients is also an essential question to answer. It is also not clear how and when the dominant clones developed and started to spread in the clinic.

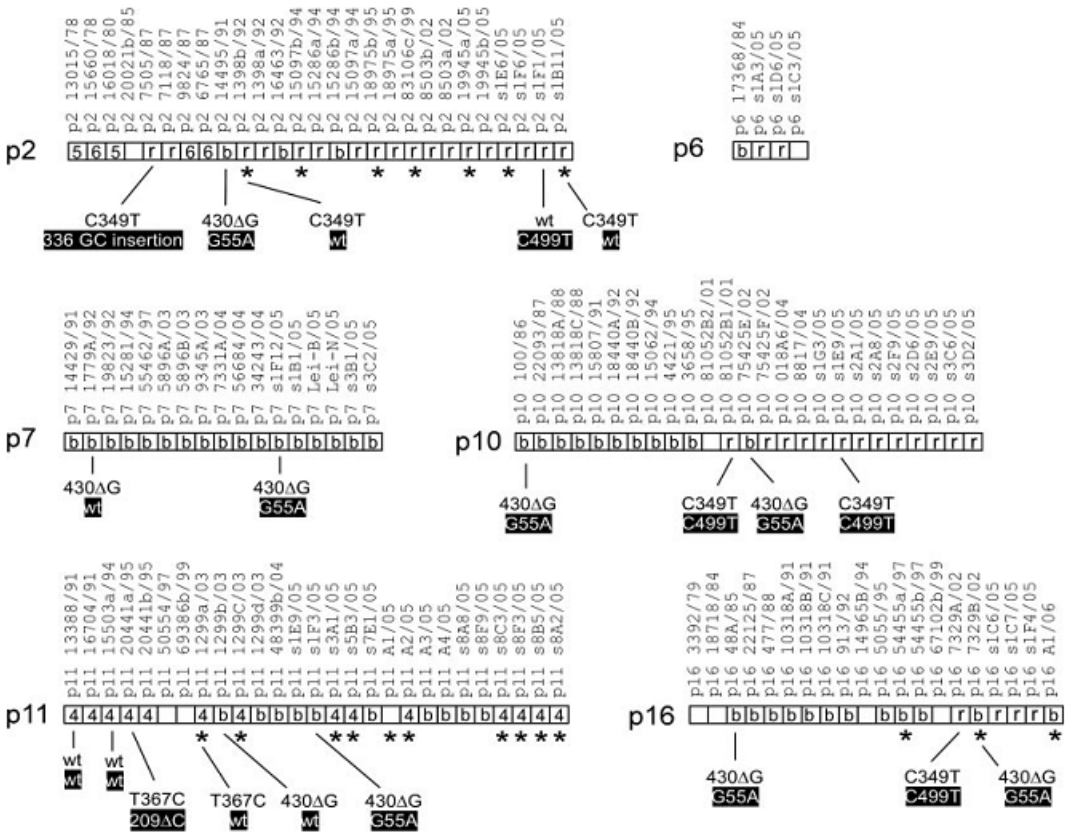


Figure 19. Genotyping of longitudinal *P. aeruginosa* isolates from patients with long-term chronic infection. Labeled boxes under the strain names specify that genotypes of the strains. White boxes specify unique genotypes found only once in the data set. Asterisks indicate mucoid isolates. The results of sequence analysis of *mucA* and *algT* compared to PAO1 from selected isolates are shown in black and white text, respectively (Jelsbak et al., 2007).

1.3.3 Inspirations from experimental evolution research on microorganisms

Considering *P. aeruginosa* infection as an evolution process in the CF lung, it is of great interest to get inspired from experimental evolution studies. Experimental evolution on microorganisms allows us to observe 'real-time' phenotypic and genetic changes in details in a relatively short time period. One of the pioneers in this field is Richard Lenski and colleagues, who initiated a long-term evolution experiments in *E. coli* (LTEE) since 1988. These experiments provided tremendous quantitative data clarifying previous evolutionary theories and addressing new insights that are still being investigated. The LTEE setting is shown in Figure 20, where twelve populations of *E. coli* B strains were propagated in a glucose limiting condition for over 40,000 generations by serial transfer. Each 24-h growth cycle allowed ~6.6 bacterial generations. Each day, the evolving cell populations experienced lag phase, exponential growth, decelerating growth, and stationary phases as the total size of

each population fluctuated between 5×10^6 and 5×10^8 cells per 10 mL culture (Lenski *et al.*, 1998). Six of the populations were founded from Ara^- that is unable to utilize arabinose as a sole carbon source, while the other six were founded from an engineered isogenic strain Ara^+. The arabinose-utilization serves as a marker in competition experiment for fitness measurement and it is selectively neutral under the selective conditions used in LTEE (Lenski et al., 1991).

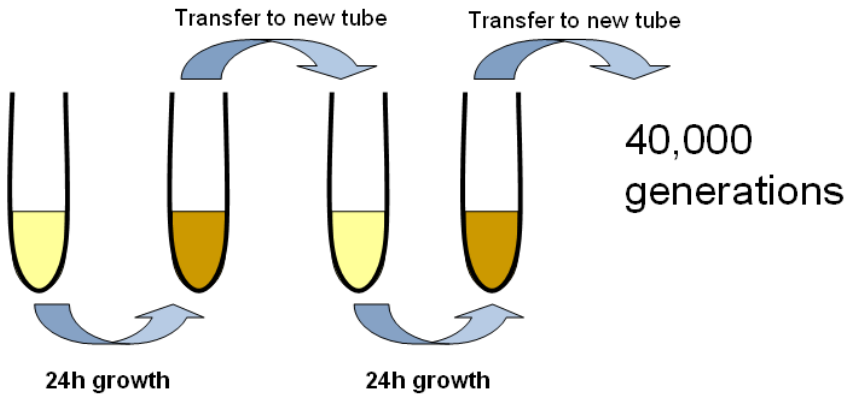


Figure 20. Long-term evolution experiments in *E. coli*. Ancestral *E. coli* strain was evolved in glucose limiting condition for more than 40,000 generations by serial transfer.

Fitness was measured by competing the evolved strain and ancestral strain in the same environment where the evolution progressed. The two competitors were mixed at 1:1 ratio, diluted 100 fold and grown for 24 h. Initial and final densities were estimated by plating cultures on tetrazolium arabinose indicator plates. The growth rate of each competitor is calculated as the natural logarithm of the ration of its final density to its initial density (adjusted for dilution). Relative fitness is defined as the ratio of growth rates of the evolved and ancestral strains (Lenski et al., 1991, Elena & Lenski, 2003). Lenski and colleagues followed the dynamics of the evolution and examined the genetic bases of the adaptation. Here we summarized some of the main observations and conclusions.

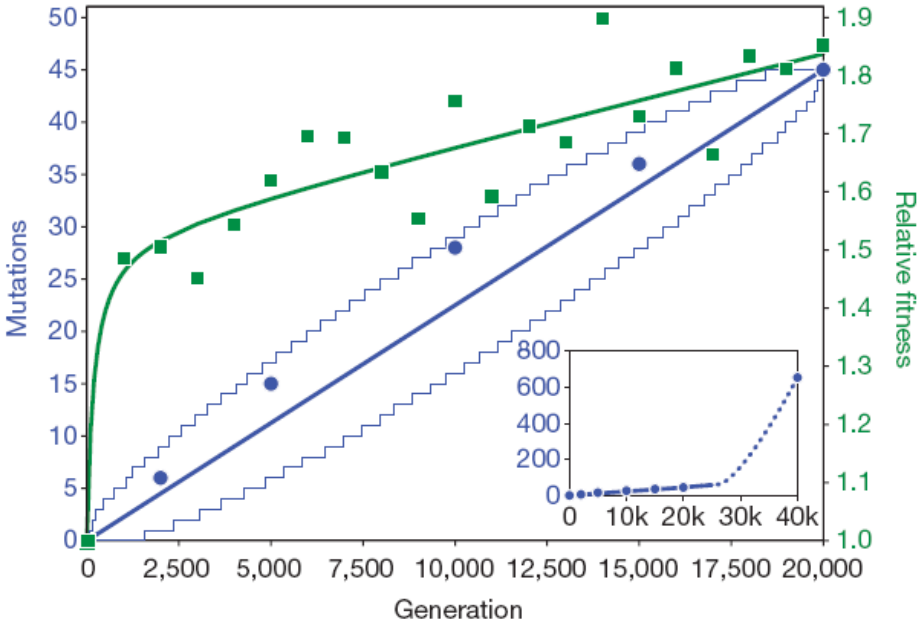


Figure 21. Rates of mutation accumulation and fitness improvement. Blue circles show the total number of genomic changes relative to the ancestor in each sampled clone. The blue line represents a model where mutations accumulate uniformly over time. The light blue curves define the 95% confidence interval for this linear model. Green squares show the increase of this population's mean fitness relative to the ancestor over time, and the green curve is a hyperbolic plus linear fit of this trajectory. Each fitness estimate is the mean of three assays; most of the spread of points around the fitness trajectory reflects statistical uncertainty inherent to the assays. The inset shows the number of mutations in the 40,000-generation clone; the dashed curve approximates the change in the timecourse of genomic evolution after a mutator phenotype appeared at about generation 26,500 (Barrick *et al.*, 2009).

Vocabulary box

Fitness: Fitness is a measurement of the ability of organisms to survive and produce offspring in the environment they live (Orr, 2009). There are many ways of calculating fitness. In experimental microbial evolutionary, fitness of the evolved strain (i) relative to the ancestor (j) is measured by competing the two for time T in the environment where the evolved strain is derived from. The population size of the evolved strain increased from N_i (0) to N_i (T) and average increase rate (Malthusian parameter) is calculated as $m_i = \ln[N_i(T)/N_i(T)]/T$.

The relative fitness of the evolved strain to ancestral strain W_{ij} is expressed as the ratio of the two Malthusian parameters, $W_{ij} = m_i/m_j$. (Lenski *et al.*, 1991, Elena & Lenski, 2003, Hansen, 2005).

Antagonistic pleiotropy: Antagonistic pleiotropy arises from trade-offs, such that the same mutations that are beneficial in one environment are detrimental in another (Cooper & Lenski, 2000).

Parallel evolution: Two or more lineages independently evolve similar or identical features (Cooper *et al.*, 2003).

Epistasis: Contribution of a mutation to an organism's phenotype depends on the genetic background in which it occurs (Cooper, 2008).

Historical contingency: Occurrence of certain mutation or phenotypic expression is contingent on prior mutations in the population (Blount *et al.*, 2008).

1. The fitness increases rapidly at the beginning and decelerate over time. This has been observed in other studies with viruses as well (Novella *et al.*, 1995, Bull *et al.*, 1997). In LTEE, *E. coli* strains gain most of the fitness in the first 2000 generations (**Figure 21**). The steep increase at the early stage of the evolution is not caused by larger amount of mutations but by very few mutations that have great impact on fitness improvement. In non-mutator strains, mutation accumulation is linear over time (**Figure 21**) (Barrick et al., 2009).
2. Evolution creates specialists which are more fit in the selective environment but have reduced fitness in other environments (trade-off). Antagonistic pleiotropy, instead of mutation accumulation, was the main cause (Cooper & Lenski, 2000). Adapted *E. coli* populations have a decay of unused catabolic functions and this happened also early in the evolutionary process, which indicates pleiotropic effects of early mutations are playing important roles. Furthermore, unused catabolic functions in mutator strains do not decay more than non-mutators, suggesting the insignificance of mutation accumulation (Cooper & Lenski, 2000).

3. Lenski and colleagues identified several same beneficial mutations occurring in many independent lineages, such as stringent response regulator *spoT* (Cooper et al., 2003). Quantitative measurement of repeatable evolution changes and statistic tests pointed towards that parallel evolution was driven by selective force, rather than random mutations (Woods *et al.*, 2006). Parallelism is strong indication of adaptive changes.

4. Mutator mutations in DNA repair system increased mutation rate dramatically (**Figure 21**) but do not seem to confer any direct fitness advantage; instead, they might spread by hitchhiking with beneficial mutations (Sniegowski *et al.*, 1997).

5. Epistasis is important in the adaptive evolution. Compared to ancestors, the evolved strains have more genes changed their expression when deleting a central regulatory gene *crp*. There is also a striking degree of parallelism in the expression profile between the two independently evolved populations (Cooper, 2008).

6. Historical contingency can contribute to innovative phenotypic changes in the evolution. A citrate-using variant (Cit^+) appeared in the late stage. Citrate is a carbon source that *E. coli* can not use; it is added in the medium for checking contaminations. However, Cit^+ variant evolved in one of the populations and extensively enhanced the fitness. 'Replay' of the evolution from different points of that population's history showed that Cit^+ did not rise before 20,000 generations, which indicate evolution of this phenotype was contingent on the particular history of the population (Blount et al., 2008).

The LTEE settings are simple and allow bacteria to evolve in a homogeneous environment. In reality, the environment such as the CF lung is much more heterogeneous and bacteria could grow as structured communities rather than homogenous suspension. All these factors facilitate to generate much more complex and diverged phenotypic and genetic changes (Rainey & Travisano, 1998). However, with coughing, DNase treatment and occasional lung flushing of the patients, bacteria in different locations in the lung are geologically possible to meet and compete. Competition and replacement of strains as well as low diversity in genotypes in *P. aeruginosa* has further supported this possibility from previous study (Jelsbak et al., 2007). This means even with complexity of the lung environment, bacteria can still grow in a relative homogenous fashion in large part of the lung and selective sweeps could still be one of the determining forces driving *P. aeruginosa* adaptation. In that sense *P. aeruginosa* evolution in CF lungs is very much comparable with *E. coli* evolution in test tubes.

We are also confronted with many problems when studying *P. aeruginosa* evolution in CF lungs. A crucial one is that we could not perform direct fitness measurement. There is no good animal model that could be used to mimic CF lungs and perform competition experiments (see 1.2.3). However, since it has been shown that parallel changes tend to have high fitness, we consider strains carrying many parallel traits and capable to transmit and out-compete others have higher fitness. One of the common changes in CF isolates is reduced virulence.

Another very challenging problem is the strain transmission. The evolution process in one patient is disturbed when transmission happens, which is indeed the case for most of the patients. Furthermore, *P. aeruginosa* in CF lungs are confronted with very complicated and fluctuating selective forces, including antibiotics, immune defence, nutrients, *etc.*, and a lot of them have not well described in molecular mechanisms. However, we hope to grab some essential clues in this project despite of all the uncertainties.

1.3.4 Objectives and Strategies

The major aim of the project is to identify and characterize the essential adaptation processes that are responsible for the chronicity of CF infection. Since we now know *P. aeruginosa* persistence involves genetic adaptation, how do we understand this microbial evolution process in the light of theories and models concluded from *in vitro* evolution? This goal will be achieved by 4 steps:

1. Determine the growth activity of *P. aeruginosa* in CF lungs.
2. Find out the overall evolutionary trajectory of *P. aeruginosa* adaptation in CF lungs.
3. Identify important genetic changes that make the dominant clones successfully adapted.
4. Investigate the diversity and parallelism of the evolutionary pathways.

Why does growth rate matter? First of all, the growth dynamics uncovers the life style of *P. aeruginosa* in CF lungs, which is the first step to understand mechanisms of the persistence. For example, tuberculosis caused by *Mycobacterium tuberculosis* is also a chronic respiratory track infection. Bacterial persistence is the result of latent stage of *M. tuberculosis* with very low metabolic state (Hu *et al.*, 1998, McCune *et al.*, 1966a, McCune *et al.*, 1966b). But in chronic gastritis infection caused by *Helicobacter pylori*, *H. pylori* has very active growth (Dubois *et al.*, 2000). Knowing the growth activity is fundamental to understand the strategies of bacterial persistence. Besides, we can use the in situ growth rates to estimate the number of generations since the first

colonization, which is obviously a key parameter when microbial evolution is what we pursue. To measure the growth activity, we used fluorescent *in situ* hybridization (FISH) to look at the localization and growth rate of *P. aeruginosa* population from sputum samples. The principle of linear correlation between ribosome content per cell volume and specific growth rate in certain range has been used to determine growth rate of samples in complex environments (Cooper, 2008, Poulsen *et al*., 1995). Thus we can use FISH to quantify the ribosome content and estimate the *in vivo* growth rate of *P. aeruginosa* both in single cell level and distribution of growth activities of the bacterial population in CF sputum samples, which represent the lungs.

To determine the important evolution pathways, systematic approaches such as whole genome sequencing, DNA microarray and Biolog Phenotype chip, *etc*. were applied and integrated data analysis was implemented to investigate the results. Here we mainly focused on one of the dominant clones, the b clone (Jelsbak et al., 2007) (**Figure 19**). Three strains will be fully sequenced: An intermittent b clone and two b type strains isolated from one chronic patient, p7, isolated in 1991 and 2007 (**Figure 19**). In this way the whole infection time from intermittent to chronic phase was covered. Transcriptome profiles during growth in defined media were obtained from the selected dominant *P. aeruginosa* isolates as well as non-adapted isolates. Here we are not focusing on the global transcriptional responses of *P. aeruginosa* strains growing in mucus (sputum) media. Instead, we search for longitudinal and parallel changes of the expression patterns related to the adaptation. This was done just in lab media. Although standard lab media cannot resemble the lung environment, most of the effects of regulatory mutations can still be tracked. On one hand, we hope to find specific pathways or functions that are essential or targeted against during the adaptation. On the other hand, how evolution shapes the overall expression profiles over time might be used as an indicator of how much the strains have adapted.

To compare what we find in genome and expression data, we also simultaneously characterized different phenotypes such as virulence factors, growth rates and antibiotics resistance patterns, as well as metabolism profiles using phenotype chips. Phenotype chip (Biolog) is also a high-through-put tool that can be used to check the growth potential on/with thousands of substrates, including nutrients, antibiotics and other toxins. We mainly focused on the catabolism of about 300 different carbon and nitrogen sources. Hopefully we could find correlations of genomes, transcriptomes and phenotype profiles in specific pathways and overall patterns related to the evolution processes.

Afterwards we tried to examine the extent of consistency and diversity of these clues in a bigger samples size. We also looked for if parallel evolution of the b clone happened in different patients to have a clearer view of how the b clone developed and spread in the whole clinic. We also compared these results with r clones using phenotypic assays and gene sequencing.

In summary, there are several features that make our project unique and especially interesting:

1. Experimental evolution with bacteria enabled us to study evolution lasting for thousands of generations. The longest experimental evolutionary study so far is the 50,000 generations of reproduction of *E. coli* in Lenski's lab initiated in 1988. The CF collection that we worked on represented the evolutionary time from 1973 until now, allowing over 150,000 generations of replication.
2. In contrast to the defined lab conditions in experimental evolution, the CF airway is a natural system with many levels of complexity. It has spatial diversity in terms of different airway compartments but also mixing of population (e.g. by coughing). It is a dynamic (e.g. fluctuation in concentrations of antibiotics) and open system with occasional environmental invaders. It also has a jungle of many other microbial inhabitants co-existing. To make things even more complicated, the detailed conditions in the CF airways, such as nutrients, are not well understood. How much we can discover about bacterial evolution in such a natural system would be extremely intriguing and important.
3. The study has a scope of large number patients in the cohort of chronically infected patients in the Copenhagen CF centre, instead of a few patients. Additionally, the history and epidemiology of *P. aeruginosa* has been well described previously (Jelsbak et al., 2007). We also have access to the information about treatment strategies, strain transmission and patient conditions.
4. Contrary to a lot of previous reports which were case stories of specific changes in CF isolates, we have employed combined systematic approaches that enable us to discover not only specific pathways that are important to the adaptation but also the relations of CF isolates in a time dependent manner.

Results produced from this project would be the first study revealing bacterial evolutionary details during chronic infection in humans. With integration of a variety of systematic approaches, we expect to provide new concepts and models of *P. aeruginosa* pathogenesis in CF infection, which could eventually lead to improved

treatment. These results would be of great significance for studying microbial evolution and other long-term infections.

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Chapter 2 *In Situ* Growth Rates and Biofilm Development of *Pseudomonas aeruginosa* Populations in Chronic Lung Infections

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In Situ Growth Rates and Biofilm Development of *Pseudomonas aeruginosa* Populations in Chronic Lung Infections▽†

Lei Yang,[1]‡ Janus A. J. Haagensen,[1]‡ Lars Jelsbak,[1]* Helle Krogh Johansen,[2,3] Claus Sternberg,[1] Niels Høiby,[2,3] and Søren Molin[1]

Infection Microbiology Group, BioCentrum-DTU, Technical University of Denmark, Lyngby, Denmark,[1] and Copenhagen CF Centre, Department of Clinical Microbiology, Rigshospitalet,[2] and Institute of Medical Microbiology and Immunology, Panum Institute, University of Copenhagen,[3] Copenhagen, Denmark

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The growth dynamics of bacterial pathogens within infected hosts are a fundamental but poorly understood feature of most infections. We have focused on the in situ distribution and growth characteristics of two prevailing and transmissible *Pseudomonas aeruginosa* clones that have caused chronic lung infections in cystic fibrosis (CF) patients for more than 20 years. We used fluorescence in situ hybridization (FISH) directly on sputum specimens to examine the spatial distribution of the infecting *P. aeruginosa* cells. Mucoid variants were present in sputum as cell clusters surrounded by an extracellular matrix, whereas nonmucoid variants were present mainly as dispersed cells. To obtain estimates of the growth rates of *P. aeruginosa* in CF lungs, we used quantitative FISH to indirectly measure growth rates of bacteria in sputum samples (reflecting the in vivo lung conditions). The concentration of rRNA in bacteria isolated from sputa was measured and correlated with the rRNA contents of the same bacteria growing in vitro at defined rates. The results showed that most cells were actively growing with doubling times of between 100 and 200 min, with some growing even faster. Only a small stationary-phase subpopulation seemed to be present in sputa. This was found for both mucoid and nonmucoid variants despite their different organizations in sputum. The results suggest that the bacterial population may be confronted with selection forces that favor optimized growth activities. This scenario constitutes a new perspective on the adaptation and evolution of *P. aeruginosa* during chronic infections in CF patients in particular and on long-term infections in general.

An understanding of the growth dynamics of bacterial pathogens within infected hosts is a fundamental issue of general biological and medical relevance and with important consequences for how bacterial infections are understood and interfered with. In cases of lifelong persistent bacterial infections, within-host growth dynamics also become an important aspect specifically related to the mechanisms of pathogen adaptation and evolution.

Chronic lung infections by the opportunistic pathogen *Pseudomonas aeruginosa* in patients with the hereditary disease cystic fibrosis (CF) are one example of a persistent bacterial infection. Most CF patients acquire chronic *P. aeruginosa* infections, and eventually these infections cannot be eradicated, even with continuous intensive antibiotic treatment (18). The infection process in the CF airways is associated with extensive genetic adaptation and microevolution of the infecting bacteria (53). The accumulation of mutations results in strains with phenotypes of which many are not usually observed among environmental isolates. These phenotypes include loss of motility (32), loss of effector proteins of the type III secretion system (19), loss of O-antigen components of the lipopolysaccharide (12, 54), reduced virulence (27, 31), reduced capacity for in vitro biofilm formation (26), and increased antibiotic resistance (8). In some cases, specific genes have been found to be hot spots for mutations during CF infections. These common targets include *lasR*, which encodes a quorum-sensing regulator (53), and *mucA*, which results in the overproduction of alginate and conversion to the frequently found mucoid phenotype (34, 35). The occurrence of a range of genetic variants during chronic infections of the CF airways and the possibility of a conserved pattern of *P. aeruginosa* mutations suggest within-host, parallel evolution of the infecting bacteria that secures their persistence and long-term survival.

P. aeruginosa is located in both the respiratory zone and the conductive zone of the infected CF lung (3, 14, 16, 25, 58). Materials from both zones can be found in sputum, which is a mixture of airway mucus, factors of the innate immune system that are induced by the infections, and other bacteria and bacterial products. The CF airway mucus forms a stationary and thickened gel which is relatively hypoxic and adherent to the epithelial lining fluid of the airway surfaces (3, 58). The airways of CF patients may thus be regarded as a complex ecological niche in which *P. aeruginosa* must adapt to challenges imposed not only from the specific physical-chemical properties of the mucus but also from inflammatory cells and molecules of the innate and adaptive defense systems, frequent

* Corresponding author. Mailing address: Infection Microbiology Group, BioCentrum-DTU, Technical University of Denmark, Building 301, 2800 Lyngby, Denmark. Phone: 45 4525 6129. Fax: 45 4593 2809. E-mail: lj@biocentrum.dtu.dk.

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‡ These authors contributed equally to this work.

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TABLE 1. Characteristics of the long-term chronically infected CF patients included in this study

Patient[a]	Yr of birth	Gender	Yr chronic infection began (no. of yr chronically infected)	Genotypes (phenotypes) of infecting *P. aeruginosa* strains[b]
p2	1966	Female	1976 (30)	r (M), r (NM)
p6	1975	Female	1984 (22)	Unique (NM), r (NM), b (NM)[c]
p7	1986	Female	1990 (16)	b (NM)[c]
p10	1963	Male	1980 (26)	r (NM)[c]
p11	1983	Male	1993 (13)	b (NM), 4 (M)
p16	1973	Female	1976 (30)	b (M), r (NM)

[a] The patients are identical to those studied in the work described in reference 22.
[b] r, b, and 4 are different bacterial genotypes that have been identified in more than one patient. Genotypes found only in single patients or found only once in the data set are designated "unique." M and NM refer to mucoid and nonmucoid isolates, respectively. For example, during the course of this study we have found mucoid and nonmucoid variants in patient p2. Both variants were of the same genotype.
[c] The bacteriology records showed that mucoid variants have been found in occasional samples (but not stored) from the history of the patients.

TABLE 2. *P. aeruginosa* strains used in this study

Strain	Source	Yr of isolation	Genotype[b]
p2 s1F6/05	CF p2	2005	r
p6 s1C3/05	CF p6	2005	Unique
p7 14429/91	CF p7	1991	b
p7 s1B1/05	CF p7	2005	b
p10 s1E9/05	CF p10	2005	r
p11 s1E9/05	CF p11	2005	b
p11 s3A1/05[a]	CF p11	2005	4
p16 s1C7/05	CF p16	2005	r
B4-1	CF B4	2005	Unique
PAO1	Laboratory		Unique
PA14	Laboratory		Unique

[a] Strain p11 s3A1/05 is a mucoid isolate.
[b] r, b, and 4 are different bacterial genotypes that have been identified in more than one patient. Genotypes found only in single patients or found only once in the data set are designated "unique."

exposure to antibiotics, and the specific nutritional conditions in the different lung compartments.

Our previous molecular epidemiological studies of the *P. aeruginosa* population dynamics among CF patients in Copenhagen, Denmark, have shown that many of the long-term-infected CF patients are infected with the same, dominant clones because of extensive cross-infections (22). On the basis of pulsed-field gel electrophoresis and single-nucleotide polymorphism analysis of conserved genes, we identified two such dominant clones, which we designated "b" and "r". In addition, we have shown that these two particular clones have been transmitted among different patients for more than 20 years (22). In order to understand better the processes that have secured the long-term persistence of these dominant and transmissible *P. aeruginosa* clones within the CF airway niche, we have focused on characterizing their in situ distribution and growth physiology using fluorescence in situ hybridization (FISH) analysis.

FISH using labeled oligonucleotide probes targeting rRNA has been a powerful technique for assessing both microbial identity (population structure) and spatial distributions in situ in complex environmental contexts (11, 15, 39, 47). Importantly, the hybridization of labeled probes to rRNA also yields a quantifiable fluorescent signal which is correlated to the cellular content of ribosomes. In several bacteria, the number of ribosomes is correlated with their growth rates (24, 38, 46, 51), and measurements of this parameter can therefore be used as an indicator of physiological activity (growth rate). The fundamental requirement for the quantification of ribosomes as a growth indicator is the establishment of a standard curve based on quantification of ribosome content in exponentially growing cultures with different growth rates (37, 49). Previous studies have used similar approaches to estimate the growth rates of bacteria colonizing different complex environments such as the mammalian gastrointestinal tract (30, 48), the rhizosphere of barley seedlings (50), and bean plant leaves (29). In the present study we have used FISH to examine the relationship between spatial organization and growth activities of both mucoid and nonmucoid cells within CF sputum specimens.

MATERIALS AND METHODS

CF patients. Data for six long-term-infected CF patients included in this study are shown in Table 1 (22). All patients were attending the Danish CF Center, Rigshospitalet, Copenhagen, Denmark. The six patients have been chronically infected with *P. aeruginosa* for more than 13 years (median, 24 years; range, 13 to 30 years). Chronic *P. aeruginosa* infection was defined as the persistent presence of *P. aeruginosa* in sputum for 6 consecutive months, or for less time when persistence was combined with the presence of two or more precipitating antibodies against *P. aeruginosa* (23). One patient, not included in Table 1, was intermittently colonized by *P. aeruginosa* (strain B4-1 [Table 2]) (22).

Bacterial isolates. The *P. aeruginosa* strain collection examined is listed in Table 2. Clinical *P. aeruginosa* strains from sputum samples were isolated on *Pseudomonas* isolation agar (Difco) containing ampicillin (100 μg/ml). All *P. aeruginosa* isolates were genotyped by single-nucleotide polymorphism typing using AT biochips (Clondiag Chip Technologies, Germany) (22).

Growth media and measurements of growth rates in laboratory media. Pure-culture growth studies of *P. aeruginosa* strains were performed under aerobic conditions at 37°C in Luria-Bertani (LB) medium; in pig mucus medium; or in ABT minimal medium (9) supplemented with either 0.2% glucose plus 1% Casamino Acids, 0.5% glucose, 2% Casamino Acids, or 10 mM sodium citrate. The mucus medium was prepared by rinsing pig lungs with 0.9% NaCl. This solution was diluted (1:1, vol/vol) in ABT minimal medium and used for growth studies. CF sputum medium was prepared by dissolving sputum samples from patient p16 in ABT (1:20, vol/vol) by vortexing for 2 min, kept at 4°C for 30 min, and sterilized by filtering through a 0.45-μm filter. All bacteria in the sputa were efficiently removed as evaluated by plating aliquots of the CF sputum medium. Anaerobic growth was performed in LB medium plus 1% nitrate at 30°C with a constant nitrogen flow. Growth rates were measured by monitoring the optical density at 600 nm during growth in 50 ml medium in 250-ml flasks with shaking at 150 rpm. Growth rates are expressed as generation times in minutes or as specific growth rates ($\ln_2$/hour [i.e., reciprocal hours]).

Isolation of bacterial cells from sputum samples. Sputum samples were processed within an hour after expectoration or stored at 4°C for later analysis. The sputum samples could be stored for more than 5 days without effects on growth rate determinations. To determine in situ growth rates, *P. aeruginosa* cells from sputum samples were extracted by dissolving the sample in 0.9% NaCl. For very thick sputa, Sputaosol (Oxoid, Hampshire, United Kingdom) was used to help dissolve samples. The samples were centrifuged at 800 × *g* for 5 min at 4°C to remove mucus and epithelial cells. The supernatant which contained bacteria was removed and fixed for rRNA hybridizations as described below.

Oligonucleotide probe. Probe PSEUDAER (5′-GGACGTTATCCCCCACT AT-3′), specific to *P. aeruginosa* 16S rRNA (21), was labeled with Cy3 (Molecular Probes, Eugene, OR). The probe has previously been reported to have a sensitivity and specificity of 1.000 (21). Potential cross-reactivity of the PSEUDAER probe is of minor concern, as the probe did not hybridize to *Pseudomonas putida* cells under the conditions used in this work (data not shown).

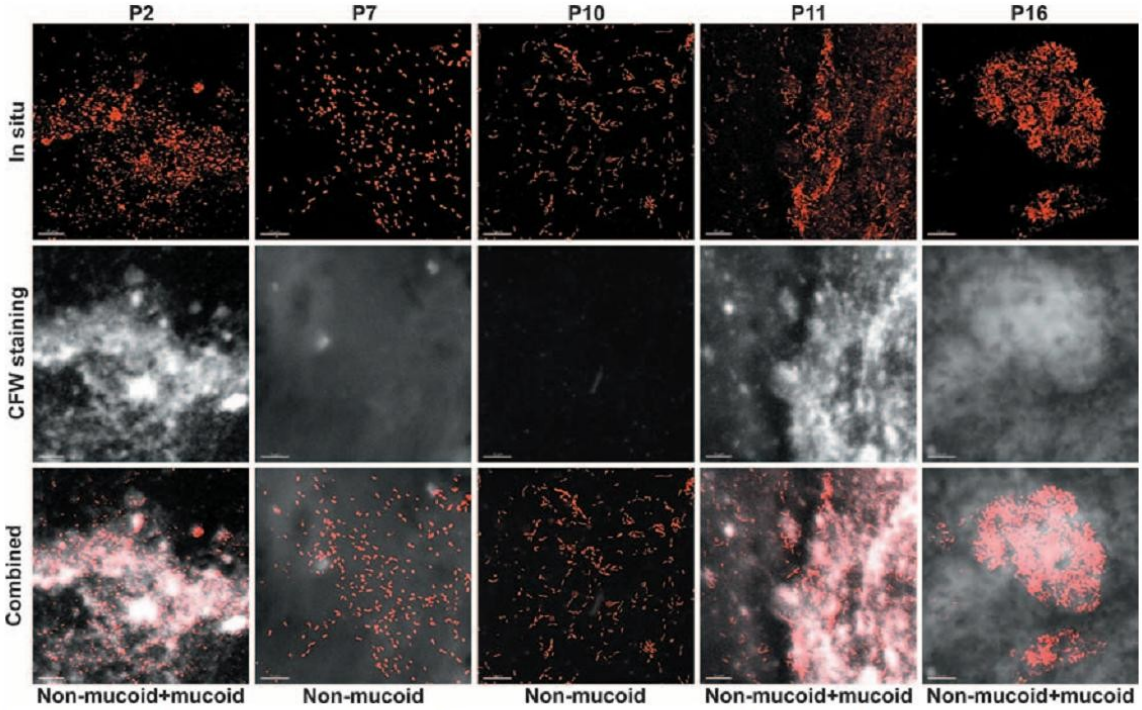

FIG. 1. Distribution of *P. aeruginosa* populations in CF sputum. Sputum samples either with exclusively nonmucoid variants or with both nonmucoid and mucoid variants from five CF patients were analyzed by CFW staining combined with FISH using a *P. aeruginosa*-specific rRNA probe. The bar in each photograph represents 15 μm.

Fixation of bacterial cells. Sputum samples, isolated cells from sputum samples, and cells from laboratory cultures were fixed in 4% paraformaldehyde for 15 min and washed with phosphate-buffered saline (PBS) (pH 7.4). Fixed cells were stored at −20°C in storage buffer (50% ethanol, 10 mM Tris [pH 7.5], 0.1% Nonidet P-40) until use.

Whole-cell hybridization. Fixed cells were applied homogenously on poly-L-lysine (Sigma Chemical, St. Louis, MO)-coated slides (36) and air dried. Hybridizations were carried out on the slides using 30 μl of solution I (30% formamide, 100 mM Tris [pH 7.5], 0.1% sodium dodecyl sulfate, 0.9 M NaCl) and 80 ng of the PSEUDAER-Cy3 probe. For FISH carried out directly on fresh sputum, hybridization solution was added directly to the sample. The slides were incubated in the dark in a humidified chamber for 3 h at 37°C, followed by washing of the slides with 45 μl of prewarmed (37°C) solution I for 30 min at 37°C and subsequently with 45 μl of prewarmed (37°C) solution II (100 mM Tris [pH 7.5], 0.9 M NaCl) for 40 min at 37°C. The slides were then quickly rinsed in distilled water and air dried. For sputum samples, 50 μl Calcofluor white (CFW) (fluorescent brightener 28; Sigma-Aldrich) was added for staining of alginate and incubated for 3 h in a humidified chamber before washing with distilled water. For FISH counterstained with the fluorescent nucleic acid dye DAPI (4′,6′-diamidino-2-phenylindole), the slides were then incubated in the dark with 14 mM DAPI in PBS at room temperature for 5 min and washed with PBS for 5 min. The slides were then rinsed and air dried.

Microscopy and image analysis. Microscopic observations of cells within sputum samples were completed using a Zeiss LSM510 scanning confocal laser microscope (Carl Zeiss, Jena, Germany) equipped with an NeHe laser as well as an UV lamp and detectors and filter sets for simultaneous monitoring of red fluorescence emitted from the Cy3 probe (excitation, 543 nm; emission filter, 565 to 615 nm) and CVW fluorescence from fluorescent brightener 28 (UV lamp excitation; emission filter, 395 to 465 nm). Images were obtained using a 40×/1.3 Plan-Neofluar oil objective. Images were processed using the IMARIS software package (Bitplane AG, Zürich, Switzerland).

Visualization of hybridized cells isolated from sputum samples or from laboratory cultures was done using an Axioplan epifluorescence microscope (Carl Zeiss) equipped with a 100-W mercury lamp and a Cy3 filter. A 63×/1.25 Plan-Neofluar oil objective (Carl Zeiss) was used for inspection and image acquisition. Image analysis was done in 12 bits with PMIS-S200 software version 4.1.4 and the Unix-based CELLSTAT image analysis program (38). Hybridized cells were automatically identified by use of the CELLSTAT program, providing cell volume, fraction of dividing cells, and mean fluorescence intensity. In some cases, unfocused cells or images and extremely high-intensity signals from crystals were deleted manually before further analysis.

RESULTS

Organization of *P. aeruginosa* populations in CF sputum. To characterize the growth physiology of *P. aeruginosa* in CF airways, we first combined FISH and CFW staining to visualize the distribution of both *P. aeruginosa* cells and exopolysaccharide materials (including alginate) directly within sputum samples (see Materials and Methods). In vitro control experiments using defined mucoid and nonmucoid derivatives of PAO1 showed that alginate was stained by CFW (data not shown). Figure 1 shows representative results from combined in situ CFW staining and hybridizations using a *P. aeruginosa*-specific 16S rRNA probe on sputum material from five CF patients. These patients have been diagnosed as being chronically infected for more than 13 years and are infected with *P. aeruginosa* clones of the r and/or b genotype (Table 1) (22). In sputum material from patients infected with both mucoid and nonmucoid clones (patients p2, p11, and p16), we observed clusters of *P. aeruginosa* cells surrounded by material stained by CFW as well as single cells in areas with only little CFW staining (Fig. 1). This finding is consistent with previous light microscopic and electron microscopic observations of cell clusters surrounded by a matrix of extracellular polymeric substances within different types of clinical specimens (including sputa) from CF patients (17, 25, 52).

In contrast, we observed mainly well-separated, single cells and little CFW staining in sputa from patients p7 and p10, who are

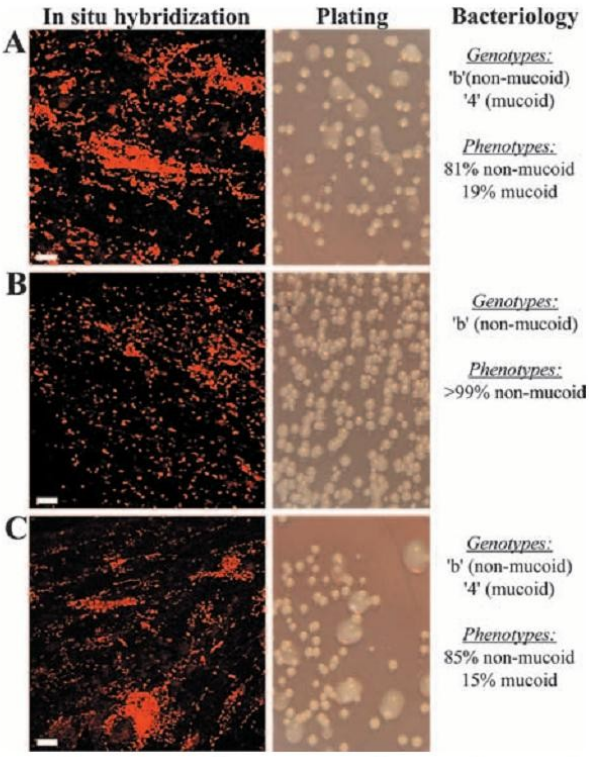

FIG. 2. Distribution of *P. aeruginosa* populations in CF sputum during a course of intravenous antibiotic therapy. Visualization of *P. aeruginosa* in sputum samples from CF patient p11 obtained before (A), immediately following (B), and 2 months after (C) an intravenous antibiotic therapy course with tobramycin and meropenem is shown. The *P. aeruginosa* population was visualized directly within sputa by FISH (left column) or by plating (middle column). The genotypes of the infecting clones as well as the frequencies of nonmucoid and mucoid variants for each time point are shown on the right. The bars represent 10 μm.

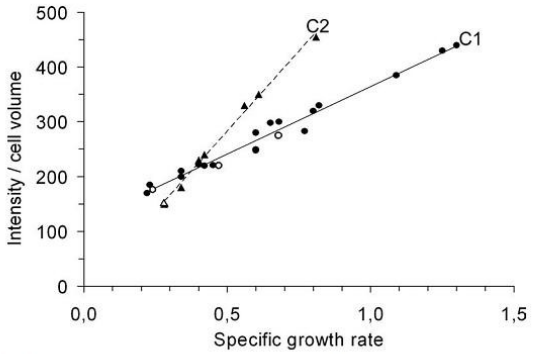

FIG. 3. Cellular content of ribosomes (fluorescence signal intensity per cell volume) inferred by whole-cell hybridization with a fluorescence-labeled 16S rRNA probe of balanced cultures grown in defined media supporting different specific growth rates. The strains and culture conditions used are described in Materials and Methods. The solid line labeled C1 and the dashed line labeled C2 are regression lines. Bacterial isolates of the b genotype (triangles) display the C2 correlation between ribosome content and specific growth rate (r^2 = 0.995). All other isolates tested (circles) display the C1 correlation (r^2 = 0.978). Key data points are highlighted by open symbols: anaerobic growth of isolate p7 s1B1/05 of the b genotype is shown by the open triangle, and open circles show growth of the mucoid isolate p11 s3A1/05 of the 4 genotype in minimal medium supplemented with (from left to right) glucose or glucose with Casamino Acids or in LB medium (fastest growth). Each measurement is the average value obtained from the analysis of the fluorescence signal from more than 100 cells. The standard error of the mean was less than 15% for all measurements. Further details related to the data set (strain information, growth media, growth rate, and intensity per cell volume) are provided in the supplemental material.

infected with only nonmucoid clones (Fig. 1). These data suggest that clones of the r and b genotypes are organized as cell clusters within sputum only when they are present as mucoid variants.

To further show that the cell clusters are composed of mainly mucoid cells, we investigated a set of sputum samples from patient p11 before and after a 2-week intravenous antibiotic therapy course with tobramycin and meropenem. Patient p11 is chronically infected with two clones, i.e., nonmucoid cells of the b genotype and mucoid cells of the '4' genotype (Table 1) (22). Routine antibiotic resistance profiling performed prior to the antibiotic course showed that the mucoid cells were sensitive to both antibiotics, whereas the nonmucoid cells were resistant. Etests of individual strains isolated before therapy showed that nonmucoid isolates had >10-fold-higher MICs for both antibiotics than mucoid isolates (data not shown). In accordance with this observation, only few mucoid cells were found in sputum samples taken immediately after the antibiotic course as evaluated by plating (Fig. 2). Importantly, this reduction in the number of mucoid cells as a consequence of the antibiotic therapy was correlated with a clear reduction in the number of cell clusters in the sputum both during and at the end of the intensive treatment period as shown by FISH analysis (Fig. 2). We note that the antibiotic treatment did not eradicate the mucoid population, as it reappeared soon after the end of treatment (Fig. 2).

Taken together, these data shows that *P. aeruginosa* cell clusters within sputum samples appear to represent mainly the mucoid population, as the clusters were not present in patients infected with only nonmucoid *P. aeruginosa* or in patients from whom the mucoid population was transiently removed by antibiotics.

The specific correlation between growth rate and cellular ribosome content varies between different clones. To be able to measure in situ growth rates of the infecting bacteria, we first determined the specific relationship between bacterial growth rate and cellular ribosome content in a series of in vitro growth experiments. To this end, we analyzed eight *P. aeruginosa* strains isolated from sputum samples from six different long-term chronically infected CF patients (Table 1). These eight isolates were of four different genotypes, and one of the isolates was mucoid (Table 2). We also included an isolate from an intermittently colonized patient (strain B4-1) as well as reference strains PAO1 and PA14. The *P. aeruginosa* strains were cultivated in different media that supported a range of growth rates. Cells were also cultivated under anaerobic conditions with nitrate as an electron acceptor as well as in a mucus medium prepared from mucus isolated from pig lungs (see Materials and Methods). In these two media, we found the doubling times of strain p7 s1B1/05 to be 149 and 104 min, respectively. During exponential growth, the cellular ribosome content was determined by quantitative whole-cell hybridization with a fluorescently labeled 16S rRNA probe.

Figure 3 shows how the cellular content of ribosomes (measured as rRNA hybridization signal intensities per bacterial

volume) increased approximately linearly with growth rates for all strains tested, as previously reported for other bacteria (30, 46, 48, 51). With the exception of strains of the b genotype (Table 2) isolated from patients p7 and p11, all other strains tested demonstrated the same specific correlation regardless of their genotype and their mucoid/nonmucoid phenotype (regression line C1 in Fig. 3). Interestingly, the linear correlation between ribosome content and growth rate for strains of the b genotype (regression line C2 in Fig. 3) was different, and the slope of the regression line was about 2 times greater than that for C1. These results show that there is a clone-specific relationship between ribosome content and growth rate and that the standard curves in Fig. 3 may be used for in situ determination of the growth activity of *P. aeruginosa* only if the genotype of the strain analyzed is known. The data used in Fig. 3 (including strain information, growth media, growth rate, and intensity per cell volume) are provided in the supplemental material.

During the in vitro growth experiments, we also observed that *P. aeruginosa* isolates obtained from long-term chronically infected patients grow two- to threefold slower than the reference strains PAO1 and PA14 in the different growth conditions tested. For example, growth of PAO1 and PA14 in rich LB medium resulted in generation times of between 24 and 27 min. In contrast, the CF isolates grew with an average generation time of 64 min, with an range of 50 to 74 min. Likewise, growth in ABT minimal medium supplemented with 2% Casamino Acids resulted in generation times of between 33 and 36 min for the reference strains, whereas the CF isolates grew with an average generation time of 119 min with a range of 103 to 129 min. The slow-growth phenotype was not an intrinsic trait of clones capable of infecting CF patients, as the growth phenotypes of six *P. aeruginosa* strains isolated from four intermittently colonized CF patients were similar to those of the reference strains (data not shown). It therefore appears that selective pressures in the CF airway environment specifically enrich for slow-growing variants.

Detection of stationary-phase, nongrowing cells by rRNA hybridization. Starved or slow-growing cells contain reduced numbers of ribosomes. To test whether fluorescence rRNA hybridization signals could be detected in starved nongrowing cells and to examine the effect of nutrient starvation on the cellular ribosome content, we transferred exponentially growing cells of the r and b genotypes as well as PA14 to ABT medium without a carbon source and measured fluorescence rRNA hybridization signals per cell volume at different time points on fixed cells counterstained with DAPI. As expected, a shift to nutrient-free medium resulted in a period of decline in ribosome content, after which the ribosome concentration remained approximately constant for at least 5 days at 50 to 40% of the level observed in exponentially growing cells (Fig. 4). Importantly, the observed significant reduction in the cellular ribosome content after only 2 h of starvation suggests that ribosomes are being degraded rapidly when cells are starved. The results also show that cells of both the b and r genotypes display similar regulation of rRNA in response to nutrient starvation, despite their genotype-specific relationship between ribosome concentration and growth rate.

After 3 days of starvation, more than 90% of the DAPI-stained cells produced detectable rRNA hybridization signals.

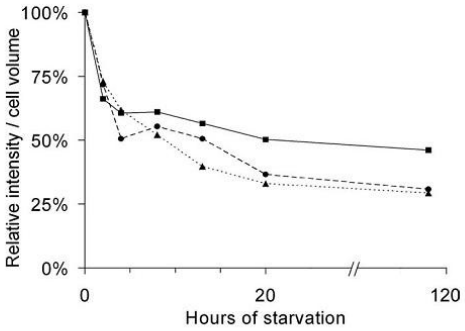

FIG. 4. Relative fluorescence signal intensity per cell volume during starvation. Exponentially growing cultures of PA14 (triangles), p2 s1F6/05 (squares), and p7 s1B1/05 (circles) in ABT plus 2% Casamino Acids were transferred to ABT medium without a carbon source. At the indicated time points, samples were taken and cellular ribosome content measured by whole-cell hybridization using a fluorescently labeled rRNA probe. The signal intensity measured prior to starvation was set to 100%. Each measurement is the average value obtained from the analysis of the fluorescence signal from more than 100 cells. The standard error of the mean was less than 15% for all measurements.

This result suggests that most starving nongrowing cells would indeed be detected in situ. As the data provide a measurement of the cellular ribosome content in nongrowing cells, it was possible to define a specific fluorescence signal intensity that clearly differentiated nongrowing, stationary-phase cells from growing cells. For each of the two genotypes analyzed, we used 120% of the fluorescence intensity measured after 20 h of starvation as the boundary separating growth from nongrowth. Cells showing fluorescence intensities lower than these values were thus considered to be stationary-phase, nongrowing cells.

Estimation of growth rates in sputum samples from CF patients. To estimate in situ doubling times of *P. aeruginosa* in CF airways, we measured the ribosome contents of *P. aeruginosa* cells in sputa from CF patients p2, p7, and p11 (Table 1). We have previously shown that p2 and p7 are chronically infected with clones of either the r genotype (p2) or the b genotype (p7) and that these particular infections are entirely clonal (22). Both mucoid and nonmucoid variants are found in p2, whereas only nonmucoid cells are found in p7. Patient p11 is chronically infected with nonmucoid cells of the b genotype and mucoid cells of the 4 genotype (Table 2). For this particular patient, we analyzed only sputum samples taken after a 2-week intravenous antibiotic therapy that targeted the mucoid population and effectively reduced the number of mucoid cells to <1% as evaluated by plating (Fig. 2). As a result, the cellular ribosome content was measured only on the nonmucoid population of the b genotype, which was unaffected by the antibiotic treatment.

Total bacteria were extracted from fresh sputum samples, and the cellular ribosomal content was measured as fluorescence rRNA hybridization signals per cell volume as described in Materials and Methods. By use of the appropriate standard curves in Fig. 3 (C1 for p2 and C2 for p7 and p11), the fluorescence signal intensities were converted to apparent doubling times.

The distribution of growth rates of *P. aeruginosa* cells isolated from a sputum sample from patient p2 is shown in Fig.

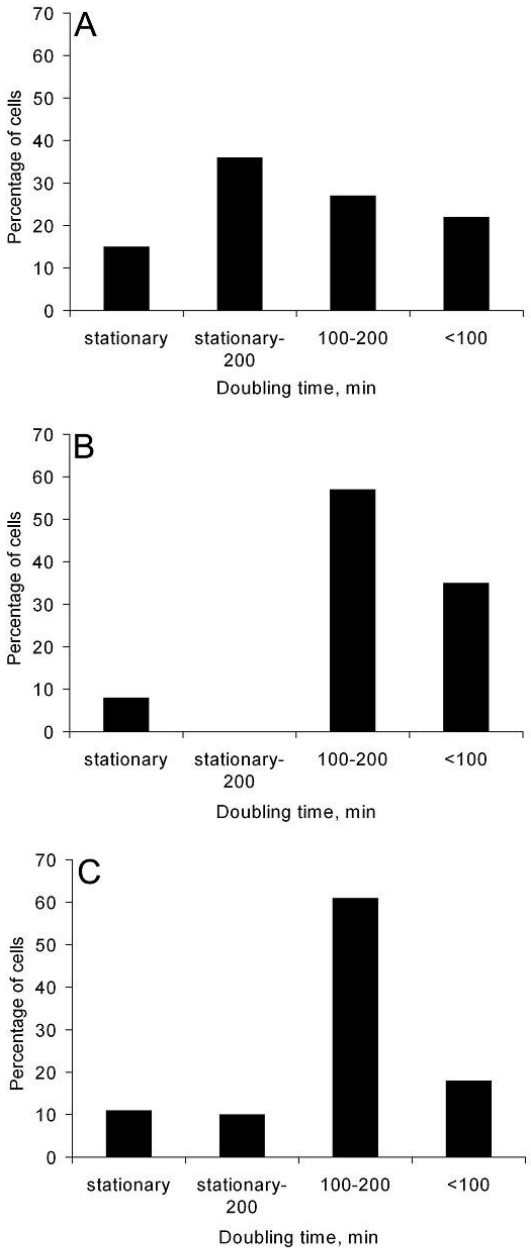

FIG. 5. Distribution of generation times of *P. aeruginosa* cells isolated from sputum samples from CF patients p2 (A), p7 (B), and p11 (C). The cellular ribosome contents (fluorescence signal intensity per cell volume) of bacteria in the samples were measured by whole-cell hybridization using a fluorescently labeled rRNA probe. These measurements were converted into apparent doubling times using the appropriate standard correlations presented in Fig. 3.

5A. Interestingly, the vast majority of the cells analyzed were found to be actively growing, and only 15% of the population was found to be stationary-phase, nongrowing cells. Most of the growing cells showed generation times of between 100 and 200 min, but a significant fraction of cells (22%) were growing even faster. Some of these cells were approaching the in vitro growth rates measured in rich LB medium for the isolated clones from this patient. The average generation time calculated for all actively growing cells in vivo was 139 min, which is comparable to the in vitro generation time in ABT plus 2% Casamino Acids.

Similar distributions of *P. aeruginosa* doubling times were observed in sputa from both patients p7 (Fig. 5B) and p11 (Fig. 5C). Again, only small fractions of the cell populations were found to be stationary-phase, nongrowing cells (8% for p7 and 11% for p11). The average generation time calculated for all actively growing cells was 115 min in p7 and 127 min in p11.

We further estimated in situ doubling times in two sputum samples with mixed populations of *P. aeruginosa*, i.e., where the samples contained more than one genotype. In a sample from p11 taken prior to the intravenous antibiotic course described above, both nonmucoid cells of the b genotype and mucoid cells of the 4 genotype were present, in an approximately 9:1 ratio. Fourteen percent of the cells analyzed were found to be stationary-phase cells, and the average doubling time for actively growing cells was found to be 134 min. In a sample from p16 which contained approximately 30% mucoid cells of the b genotype and 70% nonmucoid cells of the r genotype (Tables 1 and 2), we found that 13% of the cell population was in stationary phase and that the average doubling time for actively growing cells was 141 min. For both samples, the most conservative cutoff value for distinguishing between growing and nongrowing cells was used.

These results show that the majority of *P. aeruginosa* cells in sputum samples were actively growing with average doubling times ranging from 115 to 141 min, which is similar to pure-culture growth in ABT medium supplemented with 2% Casamino Acids. Importantly, these growth characteristics appeared to be independent of bacterial genotype, clonal versus mixed infections, mucoid/nonmucoid phenotype, and the patients from whom the sputum sample was obtained.

Pure-culture growth in CF sputum media. In order to assess the relevance of the in situ growth rate measurements, the following in vitro experiment was performed. A sterile CF sputum growth medium was prepared from a sputum sample from p16 (see Materials and Methods). Pure-culture growth of PAO1 in this medium resulted in a doubling time of 66 min, which is significantly faster growth than observed in situ for the clinical strains in p16. Furthermore, pure-culture growth of strains p16 s1F4/05 and p7 s1B1/05 in the CF sputum medium resulted in generation times of 169 min and 112 min, respectively. Thus, the two- to threefold reduction in growth rate of the clinical isolates relative to reference strains observed in vitro in different growth media was retained in the CF sputum medium. Importantly, the agreement between these in vitro results and the in vivo measurements strongly indicates that the bacteria are indeed growing at their maximum potential in the sputum.

DISCUSSION

We have examined the distribution and growth characteristics of *P. aeruginosa* colonizing the airways of CF patients. We have previously shown that many of the long-term-infected CF patients at the CF Center in Copenhagen are infected with two prevailing clones because of extensive cross-infections among the patients (22). The patients and their infecting isolates studied here are therefore representatives of a large number of chronically infected patients.

To specifically visualize the *P. aeruginosa* populations colonizing the CF patients, we used FISH with a *P. aeruginosa*-specific rRNA probe directly on sputum samples (15). Compared to light and electron microscopy methods, FISH analysis provide specific, molecular detection of the particular bacteria studied. The spatial distribution of *P. aeruginosa* cells within sputum samples was found to be dependent on the mucoid/nonmucoid status of the infecting bacteria. Clusters of *P. aeruginosa* cells surrounded by an CFW-stainable exopolysaccharide matrix (presumably alginate) were found in patients with mucoid variants, whereas these clusters as well as CFW staining were absent in sputum material from patients infected with only nonmucoid variants. In these patients, the bacteria were found mainly as well-separated cells. In addition, cell clusters were also absent in patients from whom the mucoid population was transiently removed by use of antibiotics. These results indicate that mainly mucoid variants may represent the biofilm lifestyle, which has been associated with *P. aeruginosa* infections of the CF airways (25, 52), whereas the nonmucoid variants examined here may be found as free-living cells not enclosed in an extracellular matrix. It is possible that in patients colonized with mixed populations (i.e., both mucoid and nonmucoid variants), alginate produced by the mucoid subpopulation may trap or embed the nonmucoid population in such a way that the observed cell clusters are composed of both types of variants.

We have previously shown that nonmucoid variants of the r and b genotypes are revertants of mucoid parent cells, as they have mutations in the *mucA* gene (22). We speculate that these nonmucoid revertants (containing *mucA* mutations as well as second-site suppressor mutations) have evolved alternative adaptive solutions that can substitute for alginate overproduction in order to enhance survival in the CF lung. These solutions probably include preferential colonization of sputum in the conductive zone of the airways (14) and being more resistant to antibiotics than strains with mucoid phenotypes (8).

To obtain estimates of the growth rates of *P. aeruginosa* when present in the airways of CF patients, we used quantitative FISH to measure the concentration of rRNA in a large number of single cells from individual sputum samples. Based on standard correlations between growth rate and cellular ribosome contents obtained from controlled in vitro growth experiments, these measurements were converted to estimates of in situ growth rates of *P. aeruginosa* in CF sputum. Previous studies have used similar approaches to estimate the growth rates of *Escherichia coli* and *Salmonella enterica* serovar Typhimurium colonizing mouse intestines (30, 48) and *Pseudomonas putida* colonizing the rhizosphere of barley seedlings (50).

The growth experiments performed to establish the standard correlations between growth rate and ribosome content showed that the *P. aeruginosa* isolates of different genotypes obtained from long-term chronically infected CF patients grow two- to threefold slower than laboratory reference strains in a variety of standard laboratory media, such as complex LB medium and minimal medium with different carbon sources, as well as in CF sputum medium. The observed reduced growth rate of isolates from long-term-infected CF patients appears not to be a phenomenon specifically associated with the Copenhagen CF center, since some CF isolates collected at other CF clinics also showed reduced growth rates in LB medium compared with non-CF isolates (13). These results suggest that the slow-growing phenotype may be a common feature of prolonged CF airway colonization. Most likely, the slow-growth phenotype develops as a consequence of the stressful environment where the cells experience continuous exposure to antibiotics and contacts with the cells and molecules of the immune system. For example, it is well established that chromosomal mutations that confer antibiotic resistance often have growth rate-reducing effects (1, 5, 6, 33, 40). Indeed, antibiotic resistance is a common and serious clinical problem in the treatment of chronic *P. aeruginosa* lung infections, and many of the nonmucoid isolates from long-term chronically infected patients attending the Copenhagen CF center are resistant to several antibiotics (Fig. 2) (2, 8, 20). Attempts to select for fast-growing revertants by repetitive growth of slow-growing cells in rich laboratory media were unsuccessful, which suggests that multiple compensatory mutations are required to restore the normal growth rate.

When establishing the standard correlations between growth rate and ribosome content, it also became evident that the correlations were dependent on the clone analyzed. Although the majority of clones shared the same standard correlation (C1 in Fig. 3), clones of the b genotype isolated from two different patients exhibited a different correlation (C2 in Fig. 3). More specifically, the C2 correlation indicates that cells of the b genotype contain a higher level of rRNA at specific growth rates of $>0.4\ h^{-1}$ than the other clones that follow the C1 correlation. While the molecular basis for the clone-specific relationship between ribosome content and growth rate remains unknown, we note that strains of the b genotype displayed a pattern of regulation of rRNA in response to nutrient starvation that was similar to the pattern found for strains that followed the C1 correlation (Fig. 4), which suggests that the stringent response is fully functional in the b clone. In support of this finding, sequencing of the *relA*, *spoT*, and *dksA* genes in strains of the b genotype did not reveal any mutations that would alter the functions of the encoded proteins (data not shown). RelA, SpoT, and DksA are required for normal stringent regulation in many bacteria, including *P. aeruginosa* (42, 43, 45).

The in vitro growth experiments performed in order to establish the standard correlations between growth rate and ribosome content were designed to cover a wide range of growth conditions that supported different specific growth rates. These growth conditions included both aerobic and anaerobic growth in defined media as well as growth in complex media such as LB and in mucus purified from pig lungs. The important conclusion from quantitative FISH measurements of cells under these diverse growth conditions is that the ribosome content depends only on the actual growth rate (and the particular clone analyzed) and not on the specific medium used. In other words, all points related to the quantitative hybridizations fall on the standard curves presented in Fig. 3. For example, strain p7 s1B1/05 was found to grow with a doubling time of 149 min both during aerobic growth in ABT plus 10 mM sodium citrate and during anaerobic growth in LB medium. In these two cultures, we also measured similar ribosome contents (150 and 153 units of fluorescence intensity per cell volume, respectively).

The in vitro starvation experiments performed to measure

the effect of nutrient starvation on the cellular ribosome content clearly showed that cells responded immediately (within 2 h) to starvation by rapid degradation of ribosomes. This means that the actual cellular ribosome concentrations reflect quite well the actual physiological states of the cells. If, in contrast, the ribosomes had turned out to be stable after the onset of starvation we would not have been able to distinguish between growing cells and stationary-phase cells with a nondegraded excess of ribosomes. Moreover, the data show that the reduced ribosome contents in cells subjected to prolonged starvation were still efficiently detected by FISH. The main conclusion from this experiment therefore is that the rapid reduction in ribosome content to a low but detectable level in response to starvation allowed determination of the fraction of stationary-phase, nongrowing cells in the sputum samples.

Our estimates of growth rates of *P. aeruginosa* in sputa by using quantitative FISH are, to the best of our knowledge, the first in situ description of the growth physiology of *P. aeruginosa* infecting CF airways. The experiments revealed remarkably similar results for three different CF patients infected by two distinct clones (Fig. 5) as well as for CF patients infected by both mucoid and nonmucoid strains of different genotypes. The observation that there were similar average doubling times in vivo for bacteria with different genotypes, different mucoid/nonmucoid phenotypes, and different correlations between growth rate and ribosome content, as well as for bacteria isolated from different patients with both clonal and mixed infections, suggests that the growth properties of the infecting strains have been optimized to the lung environment during long-term colonization and that these particular conditions are very similar in different patients. In this view, the reduced growth rates of the different clinical isolates may be regarded as an example of parallel evolution of an adaptive trait, analogous to the parallel adaptation of independent bacterial cultures to a defined laboratory environment during long-term evolution experiments (10, 28, 44).

The average doubling times calculated for actively growing cells in sputa from these patients were in all cases comparable to the doubling times measured in ABT minimal medium supplemented with 2% Casamino Acids in controlled in vitro growth experiments as well as to doubling times measured both in pig mucus media and in CF sputum media. High levels (15 to 20 mM) of amino acids have been observed in CF sputum (4, 56), and recently a transcriptome analysis of *P. aeruginosa* cells growing in medium made from CF sputum strongly suggested that amino acids within the sputum indeed are the likely candidates as carbon and nitrogen sources for the bacteria (41). Our data suggest that ABT minimal medium supplemented with 2% Casamino Acids, pig mucus medium, and CF sputum medium all may be appropriate growth substrates for in vitro studies of *P. aeruginosa* isolates from CF patients.

The distributions of in situ doubling times indicate that most cells are actively growing cells. Only 8 to 15% of the cell populations analyzed were characterized as being nongrowing cells (Fig. 5). We recognize that some inaccuracy exists in the discrimination between growing and nongrowing cells. For example, some nongrowing cells may have a hybridization signal too faint to be recorded. However, in control experiments with DAPI-counterstained starving cells, we found that a faint hybridization signal may result in only a slight underestimation of the number of stationary-phase cells. Also, the hybridization signal chosen for the differentiation between growing and nongrowing cells may contribute to some inaccuracies. We chose 120% of the hybridization signal value after 20 h of starvation as the cutoff value for growing cells, which is a highly conservative choice and may in fact result in significant overestimation of the number of nongrowing cells. Nevertheless, we consider the contribution of these potential errors to be insignificant in relation to our major conclusion that the clear majority of *P. aeruginosa* cells are actively growing in the airways of the CF patients.

The finding that most cells in vivo are actively growing at relatively high growth rates has implications for the understanding of the *P. aeruginosa* population dynamics and organization in chronic CF airway infections. First, the result suggests a high rate of turnover of bacterial biomass. This turnover is most likely caused by antibiotic treatment, immune system attacks, and removal of biomass by coughing. Second, the results indicate that the bacteria may not be organized in the same type of biofilms as described from in vitro investigations. Laboratory-based flow chambers and related setups (7) have been used to mimic and study the biofilm lifestyle, which for a long time has been linked to *P. aeruginosa* infections in CF patients (3, 14, 16, 25, 52). However, biofilm development in vitro most often results in spatial patterns of growth activity with the majority of the population being inactive, nongrowing cells located in the center of the biofilm and a subpopulation of active, rapidly growing cells near the nutrient- and oxygen-exposed surface (55, 57). We performed quantitative FISH analysis on cells of PAO1 and p7 s1B1/05 isolated from 3- and 8-day-old in vitro biofilms. For both strains, more than 45% of the cells were found to be nongrowing, stationary-phase cells using the same analysis as carried out on cells from sputum (data not shown). We therefore argue that the growth physiology of cells from in vitro biofilms may be incompatible with the in vivo growth activities measured here and that the absence of heterogeneity with respect to growth activities in sputum indicates a lifestyle characterized by growth of dispersed, single cells. In fact, we did observe that nonmucoid cells were organized mainly as dispersed, single cells in sputum (Fig. 1). On the other hand, mucoid variants were found to be organized in clusters surrounded by an extracellular matrix in sputum. However, as the majority of these cells were actively growing cells, we suggest that the cells are distributed within these clusters in such a manner that internal heterogeneities (e.g., gradients of nutrient availability) may not interfere with growth. We observed that the cells within in vitro biofilms formed by the mucoid isolate p11 s3A1/05 were not as densely organized as observed for PA01 in vitro biofilms (data not shown). This suggests that microenvironments that impose constraints on growth activities may not develop in situ, as the mucoid cells are essentially growing as well-dispersed cells within an extracellular matrix.

In conclusion, we propose that the complex CF airway environment impose two opposite selection forces on the *P. aeruginosa* populations that chronically infect CF patients. On one hand, there is a clear selection for clones with highly reduced growth rates. It remains to be seen if slow growth per se is the selected property or if slow growth is a consequence of other selected properties such as increased antibiotic toler-

ance. On the other hand, competition for nutrients favors relatively fast growth of the bacteria within the CF airways. This, in turn, may be incompatible with the biofilm mode of growth as defined from in vitro settings. This model constitutes a new perspective on both the adaptation and evolution of infecting *P. aeruginosa* populations but also on the within-host growth dynamics of the bacteria during chronic lung infections in CF patients. An understanding of both aspects has important consequences for the design of new preventive measures.

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Table S1. Growth rate and fluorescence data used in Figure 3

Standard curve	Strain	Genotype	Medium	Specific Growth rate (h^{-1})	Intensity/ Cell Volume
C2	p7 s1B1/05	b	LB	0.61	350
			ABT+1%casamino acids and 0.5%glucose	0.42	240
			pig mucus medium	0.4	230
			ABT+10mM Sodium Citrate	0.28	150
			anaerobic growth	0.28	153
	p7 14429/91	b	LB	0.81	455
	p11 s1E9/05	b	LB	0.56	330
			ABT+1%casamino acids and 0.5%glucose	0.42	240
			ABT+10mM Sodium Citrate	0.34	180
C1	p11 s3A1/05	4	LB	0.68	275
			ABT+1%casamino acids and 0.5%glucose	0.45	221
			ABT+10mM Sodium Citrate	0.24	176
	B4-1	unique	LB	1.09	385
			ABT+10mM Sodium Citrate	0.77	283
			ABT+0.2%glucose	0.6	248
	p16 s1C7/05	r	LB	0.65	298
			ABT+1%casamino acids and 0.5%glucose	0.4	222
			ABT+10mM Sodium Citrate	0.23	185
	p6 s1C3/05	unique	LB	0.68	300
			ABT+1%casamino acids and 0.5%glucose	0.34	210
			ABT+10mM Sodium Citrate	0.22	170
	p2 s1F6/05	r	LB	0.6	280
			ABT+2%casamino acids	0.42	220
			ABT+10mM Sodium Citrate	0.34	200
	PA01	unique	LB	1.3	440
			ABT+10mM Sodium Citrate	0.8	320
			ABT+0.2%glucose	0.6	250
	PA14	unique	LB	1.25	430
			ABT+10mM Sodium Citrate	0.82	330
			ABT+0.2%glucose	0.47	220

Chapter 3 Paleontological study of 150,000 generations of *Pseudomonas aeruginosa* evolution in CF lung environment

Paleontological study of 150,000 generations of *Pseudomonas aeruginosa* evolution in CF lung environment

Lei Yang[1], Lars Jelsbak[1], Søren Damkiær[1], Christopher Workman[1], Rasmus L. Nielsen[1], Martin H. Rau[1], Susse K. Hansen[1], Helle K. Johansen[2], Niels Høiby[2], Søren Molin[1*]

[1]Department of Systems Biology, Technical University of Denmark, 2800 Kgs. Lyngby, Denmark

[2]Copenhagen CF centre, Department of Clinical Micorbiology, Rigshospitalet, Juliane Mariesvej 22, 2200 Copenhagen, Denmark

[*] Corresponding author: Department of Systems Biology, Technical University of Denmark, Building 301, 2800 Kgs. Lyngby, Denmark. Email: sm@bio.dtu.dk

3.1 Abstract

Microbial evolution studies in defined conditions have contributed significantly to our knowledge of the genetic basis of evolution. However, microbial evolution process occurring in a natural environment has never been reported due to the obvious complexity. Here we describe a unique case of long term microbial evolution in a natural environment – genetic adaptation of *Pseudomonas aeruginosa* in cystic fibrosis chronic infection since 1973. Thanks to an exceptional collection of sputum isolates derived from the Copenhagen CF clinic, it has been possible to follow the evolutionary process over a period of 35 years corresponding to more than over 150,000 generations of bacterial growth. We focused on one cell-line, the lineage b that has been dominating and transmitting in many patients. Through systematic analysis of transcriptome data, genome sequences and metabolic profiles, we could estimate the evolutionary trajectory of this *P. aeruginosa* clone in chronic CF airways. The development of the b clone from the early stage of the infection to highly adapted stage has displayed a step-wise progression, in which major changes happened early in the evolution and these changes were mainly caused by very few mutations in regulatory genes. This early rush of the adaptation has allowed this particular clone to out-compete environmental strains and become dominant in many patients. All the different highly adapted *P. aeruginosa* exhibited similar patterns in terms of transcriptional and metabolic profiles, but distinct from early or environmental isolates. Besides, certain parallel evolution traits were also identified in phenotypic and genetic levels.

3.2 Introduction

Studies of evolution on microorganisms have received increasing attention, not only because many microbes develop close relations (beneficial or pathogenic) with human beings, but also because microorganisms have great advantages for evolution experiments which allowed us to record on-going details of evolutionary progress which could shed light on the understanding of evolution in general. One of the pioneers in this field is the group led by Richard Lenski, who propagated 12 *Escherichia coli* cultures for more than 40,000 generations in glucose minimal medium since 1988 (Lenski *et al.*, 1991). From this long-term experiment, many fundamental questions of evolution process have been clarified and previous phenotypic observations have been quantified with genetic basis, including kinetics of fitness increase, examples of parallel evolution, prevalence of adaptive changes in global

regulatory networks, *etc*. (Cooper *et al*., 2003, Cooper & Lenski, 2000). However, these investigations were performed with isolated pure culture evolving in defined conditions. Test tubes are far from mirroring the complexity of natural environments. It is thus a very intriguing challenge to uncover the mechanisms of microbial evolution in 'real-world' cases, and assess the compatibility of these with current evolutionary theories drawn from experimental evolution studies.

The specific model we chose was airway infection of the opportunistic pathogen *Pseudomonas aeruginosa* in cystic fibrosis (CF) patients, an infection which originates early in childhood and remains in patients for the rest of their lives. It is widely accepted that CF patients often acquire *P. aeruginosa* strains from various environmental reservoirs and the bacteria persist in CF airways through considerable genetic adaptation (Smith *et al*., 2006). The time point of the first acquisition of *P. aeruginosa* defines a reference point for all subsequent evolutionary changes as long as the same clone resides in the patient. In analogy with known examples of laboratory based evolution experiments, the chronic CF infections offer similar possibilities of sampling and characterization of the developing populations. The complexity of the patient airways (including the therapeutic measures taken by the medical staffs) is obviously far greater than the usual laboratory conditions and not well understood at the molecular level. However, the confinement of the bacteria to individual airway compartments in specific patients provides parallel opportunities for analysis of adaptive processes compared with those determined by laboratory settings.

Although bacteria often have very short generation times allowing investigations of adaptation over hundreds or even thousands of generations, more substantial evolutionary changes may require tens of thousands or hundreds of thousand generations of growth. In many ways this reflects the general problem of many evolutionary studies, which have only been possible through findings of fossils in samples of known age. Here we took the advantage of a unique historical strain collection that contains huge amount of *P. aeruginosa* isolates from hundreds of patients in the Copenhagen CF clinic with 35 years of time span. Given that *P. aeruginosa* in chronic patients has a doubling time of 100-200 min on average (Yang *et al*., 2008), lapses of 35 years allow more than 150,000 bacterial generations, which concedes immense potential for genetic alterations. This collection covers the entire course of *P. aeruginosa* evolution since its early transition from environment to human lung until very late stage of the infection in which *P. aeruginosa* has highly adapted to the lung.

The population dynamics of *P. aeruginosa* in these patients have been thoroughly investigated and two distinct and dominant clones were identified in chronic patients (Jelsbak *et al.*, 2007). The evolutionary triumph of these two clones was manifested by the fact that they have been able to successfully persist and transmit among patients for more than 2 decades (Jelsbak et al., 2007). The present study focuses attention on one of the 2 dominant clones. We applied systematic measurements of transcriptional and metabolic profiles of isolates with this genotype from different patients and demonstrated the portrait of a successful persister. Together with genomic mapping of specific targets, we managed to estimate the evolution trajectory of this clone in many CF patients over time and determine some of the crucial steps. Other aspects concerning parallel evolution were also illustrated in details.

3.3 Results

3.3.1 First glance of the evolutionary consequence: profound remodelling and loss of ID

If bacteria migrate from natural environments (aquatic, terrestrial) to human internal environments in which they adapt over time, it is expected that the extensive switch in conditions will lead to substantial changes in life-style caused by numerous adaptive mutations. We assessed this expectation by performing diagnostic identification tests on a number of isolates of *P. aeruginosa* derived from different time points in infection in CF patients. Strains obtained from very early time points were unequivocally identified as *P. aeruginosa* using Biolog ID plates (see Material & Methods), whereas isolates with a history of more than 15 years of human airway colonization could not be identified at the species level (in some cases not even at the genus level). Thus, the overall phenotypes of isolates from chronically infected patients drift away from the normally conserved species specific profile, even though the genetic changes in the different cell lines may not be the same. This confirmation of the expected genome changes will be explored further in the following text.

3.3.2 Our 'fossils' – strain collection

In 1973 a 'fossil' collection of *P. aeruginosa* isolates from CF patients was initiated at the CF Clinic in Copenhagen, and subsequently isolates from many patients have been deposited in this collection, which today comprises many thousand strains. In the beginning the strains were stored as agar slants, later kept frozen at -80°C in glycerol. Like any fossil collection the CF isolates in the Copenhagen CF Clinic do not

constitute a complete coverage of all stages of evolutionary development. Some of the isolates (mainly the earliest ones) were not kept under optimal conditions and mutations may have been introduced during storage. There are gaps in the collection which represent 'black boxes' of our investigation. The individual isolates represent single clones isolated from patient samples after growth on laboratory substrates and may or may not represent dominant subpopulations of the infecting bacteria; in all these cases the population diversity in the patient airways is unknown. Despite these and other problems we think that this CF strain collection is unique and sufficiently complete and representative for conclusive investigations of the evolutionary trajectories of some the bacterial cell lines which developed in the corresponding patients.

We showed previously that at least two different genotypes (named b and r) were isolated from a large number of patients suggesting that transmission of bacteria had occurred among the CF patients in Copenhagen. One of these, the b clone, is the target of the present investigation. Based on a combination of PFGE and AT Chip analysis we found that out of 161 chronically infected patients who have been treated as one cohort, 42 had been infected with the b strain and many of them were carriers of the b strain over longer time periods. Among 40 isolates from 1973 (the oldest isolates in the collection) 4 were identified as b. Finally, a clone was isolated in 2007 from a CF patient with a *P. aeruginosa* airway infection (first and last time for this patient) which turned out to be very closely related to the b clone. In a separate project we have monitored all colonizing clones of *P. aeruginosa* sampled from CF children and other patients with early infections in the Copenhagen CF Clinic during the last 5 years, and in no case except the one described above have we identified the b genotype. We therefore conclude that the b clone is present in the larger Copenhagen environment, but it does not seem to be a dominating genotype. The hypothesis that the b isolates in the clinic are descendants from a very early infection rather than new infections from the environment is supported by the observation that all b isolates except the 2007 strain described above share phenotypes with other chronically infecting strains (slow growth, antibiotic resistance, loss of, *etc.*, see **Table S1**). In contrast to the well-adapted b isolates in older patients which cannot be cured, the b strain isolated from the newly colonized CF patient was successfully cured by antibiotic treatment and has not been seen since.

In relation to the b clonal line of isolates we therefore assume that i) it has been present in CF patients of the Copenhagen CF Clinic since the early 1970s, ii) it has

been transmitted among many patients such that 20-25% of the chronically infected patients have been infected with the clone, and iii) a closely related clone with a very short recent infection history has been obtained. Due to the short period of colonization of the latter b like clone we consider it a putative wild-type environmental relative of the b adapted isolates in the CF patients. As such we have used it as a reference strain (referred to Wild-Type-b or wtb) for our detailed genotypic and phenotypic investigations.

3.3.3 Evolutionary progression reflected by expression profiles

To further characterize the adaptation of the b clone in the Copenhagen CF clinic, we first performed systematic comparison by DNA microarray analysis in 14 chosen isolates as well as PAO1. Figure 1 shows the clonal dynamics of *P. aeruginosa* isolates in the patients who we focused on. There were 4 isolates with the b genotype found in 1973 isolated from 4 different patients. The b strains did not persist long in any of them, due to the fact that the patients died (CF42, CF112 and CF105) or strain substitution (CF66). In the case of CF66, after 1973 b isolates did not appear until 1992, but phenotypes of the isolates after 1992 bear more resemblance to other late isolates than to the one from 1973, indicating that the patient was re-infected with a b strain that was already adapted to the lung through transmission. Two isolates were used for expression profiling – CF43-1973 which is similar to environmental strains in phenotypes (**Table S1**) and CF66-1973 which is related to other later isolates in several gene sequences (**Table S2** and following section). Patient CF30 carried the b genotype in 1979 once and was then dominated by the r clone afterwards. The CF30-1979 isolate is the only one available for us between 1973 and 1984. Patient p7 is especially interesting because a very homogenous 'b clone only' pattern is found in the strain collection ever since 1991 when she was just diagnosed as chronic patient. Longitudinal samples from 1991, 1997, 2003, 2005 and 2007 were chosen for transcriptome analysis. Patients p6 and p10 also have a similar 'b clone only' pattern for a long time as p7, except they both experienced transition to r genotype in 2002 for p10 and 2005 for p6. Three isolates from 1984, 2002 and 2005 of p6 and a pair of r and b isolates from the same sputum sample of p10 in 2002 were chosen in the DNA microarray analysis. The strain wtb and PAO1 were used as environmental references. These isolates covered the whole period since b genotype was first found in the clinic in 1973 until year 2007 and contained diversity in terms of patients and genotypes.

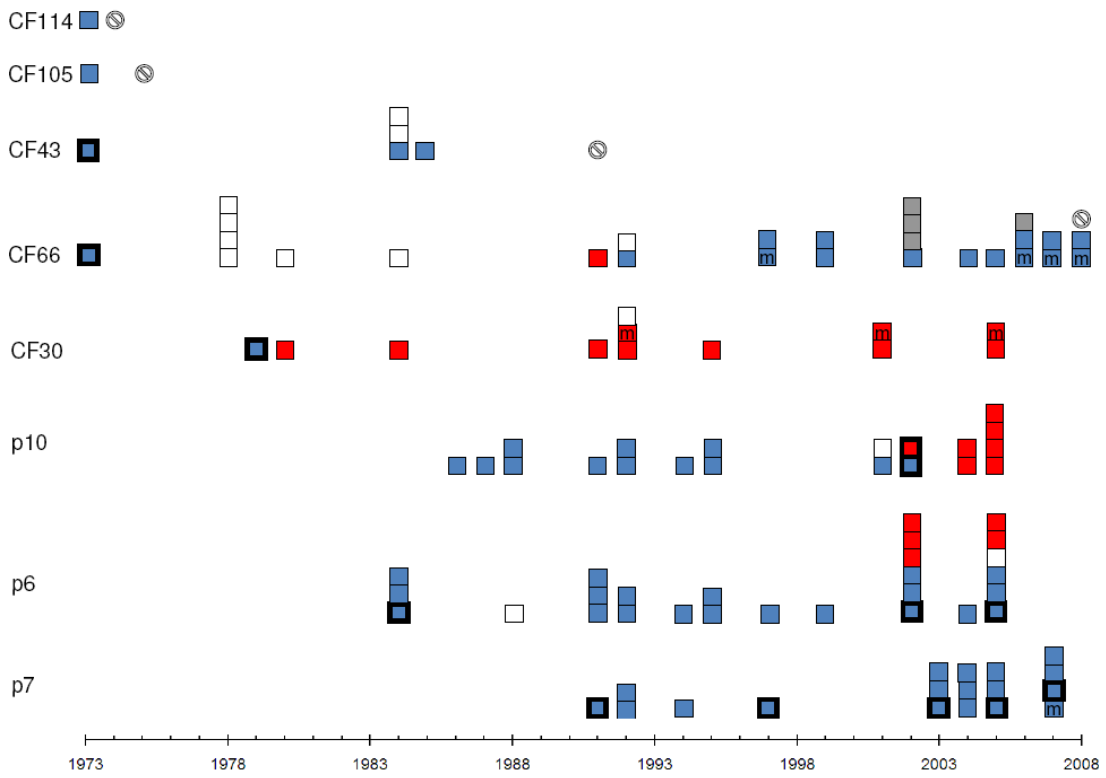

Figure 1. Strain dynamics of focused patients. Blue box: b genotype; red box: r genotype; White box: other genotypes neither b nor r; Gray box: genotyping not performed; 'm' inside box indicates the mucoid phenotype (alginate production); ⦸ indicates dead patient. Bold box specify the strains that were used for transcriptome and metabolic profiling analysis.

To obtain an overview of the microarray data, we performed principle component analysis (PCA) using singular value decomposition (SVD). The expression level of each gene is the mean values of 3 independent experiments (5 replicates in case of strain wtb). All the 5900 probes on Affymetrix Genechip PAO1 were used for calculation. PCA allows the visualization of data of 15 points in a space of 5900 dimensions (a matrix of 15 strains and 5900 genes) in a two dimensional space. Figure 2 shows the PCA plot in which the variance is projected on the first two principle components (SVDs) which captured the most variation between strains, 34% and 18% respectively. Surprisingly all the CF isolates harvested after 1979 form a large cluster, far way from environmental strains wtb and PAO1. The two isolates from 1973 were located in the between the above two big clusters. There are at least 3 important pieces of information that can be extracted from this figure. First, the cluster of late isolates contains strains from 4 different patients and 2 different genotypes, but they all behave with high level of homogeneity. This points towards that there are common evolutionary traits driven by the similar selective forces in CF lungs. Second, the late CF isolates are very distant from their environmental relative wtb, suggesting

that expression profiles of CF isolates have been deeply reshaped by genetic adaptation during the long term persistence. Third, projections of all the strains on the first SVD have a strikingly nice correlation with the isolation year. From right to left, we have environmental strains, 1973 isolates, 1979 isolate and later isolates, although the strains harvested after 1979 did not show strong time-dependent trend.

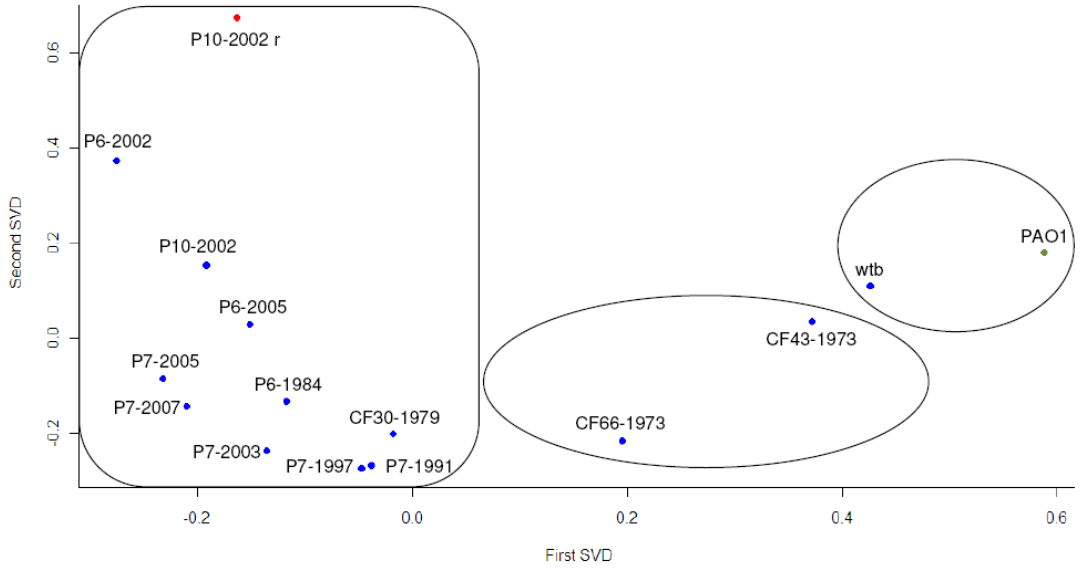

Figure 2. Two-dimensional SVD plot showing the relationship between different isolates. Genotype was indicated by colours. Blue: b; Red; r; Green: PAO1. In the first two SVDs that capture highest percentages of variance (34% and 18%), all the strains fall into 3 major clusters: environmental representatives (PAO1 and wtb), intermittent isolates (1973 isolates) and all the late isolates.

To further investigate this time-related correlation, we made a one-component analysis where the loadings of the first SVD are extracted and projected against the isolation years (**Figure 3**). We can see an unambiguous decline over time. The trajectory declined rapidly before 1979 and became flattened afterwards. Among the two 1973 isolates, CF42-1973 is more similar to wtb, while CF66-1973 is closer to the late isolates, which also agrees with the phenotypic data that CF66-1973 showed reduction in growth rate and motility. All the isolates after 1979 are relatively more similar to each other. However, if we zoom in to specific patient, we can still see a gradual progression. In p7 who has b clone persistence exclusively, 5 longitudinal isolates displayed a stepwise decreasing in their loadings in the first SVD, implying that most likely no strain transmission happened in the patient since 1991. In contrast to the consistency of p7 isolates, p6 strains showed a reduction in first SVD loadings from 1984 to 2002, but the trend reversed from 2002 to 2005, when strain transition

just occurred. This reflected a more complex pattern in the infection history of this patient, which could be the consequences of possible strain transmission, change of therapy, colonal diversity, *etc*. The isolate p10-2002 r (r genotype) also has similar first SVD loading with all the late b isolates although it has a different genome backbone, strongly suggesting the parallelism of evolution of *P. aeruginosa* infection in CF chronic patients.

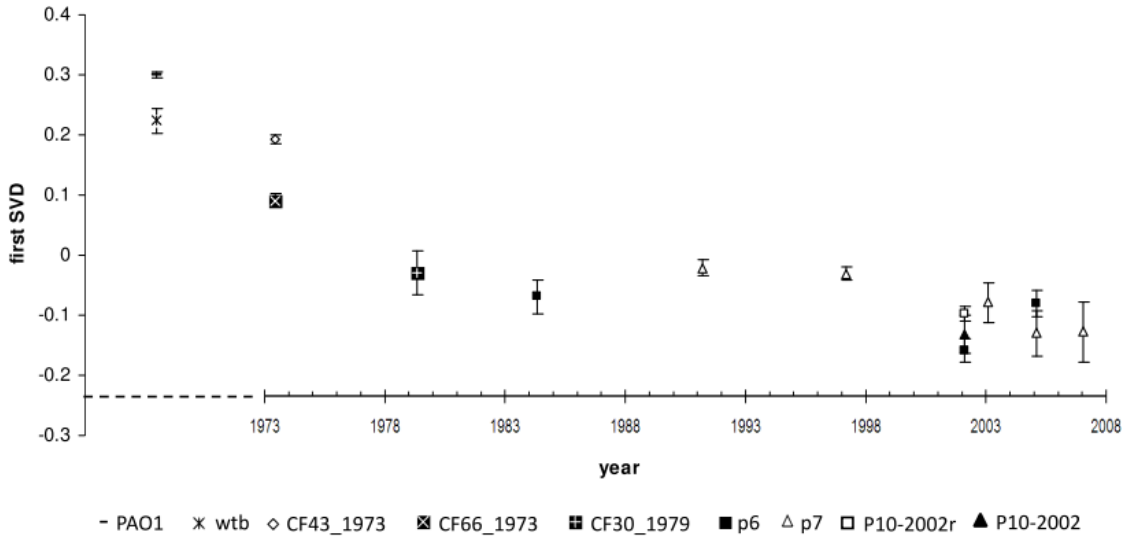

Figure 3. Time dependent progression of b isolates. Loadings of the first SVD were plotted against time. Error bars indicate standard deviations of the three replicates.

To analyze the causes of this time-dependent progression, we extracted the top 2% genes that contribute the most to the first SVD, which are the genes that could best elucidate the change of transcriptional profiles over time, listed in Table S3. Interestingly, we can see many changes in gene expression levels correlating well with what we see in phenotype alterations. For example, there is decline in Quorum Sensing (QS), reduction in motility and increase in resistance to colistin from environmental isolates to early CF isolates and further to late CF isolates, and these can be explained in mRNA level by down-regulation of *lasI*, *rhsI*, *flg/fli* genes, and *pilA*, and up-regulation of *arnB* operon respectively. The next question is whether we can reveal the genetic alterations in regulatory genes that cause these expression changes.

3.3.4 Evaluation of important mutations in *mucA*, *lasR* and *rpoN*.

An apparent trend in Figure 2 and Figure 3 is that the expression profiles of the b clones changed dramatically from 1973 to 1979 but remained relatively stable after 1979. One plausible explanation for this deceleration is that early mutations occurred

in global regulator genes which have essential benefits for the adaptation as well as numerous pleiotropic effects and thus have more influences in the transcriptional profiles. The later mutations involve only local alterations and small steps of adjustments, such as compensatory mutations to restore the deleterious side effects of previous mutations, or different switches in antibiotic resistance mechanisms in accordance to the change of therapies in patients. There are 3 candidate genes that have the properties of carrying both beneficial and pleiotropic effects, *mucA*, *lasR* and *rpoN*. The genes *muc*A and *lasR* had always been celebrities in CF study because they are frequently mutated in CF isolates. Elimination of *mucA* has potentially advantageous functions relating to the stress response (through derepression of AlgU) and virulence attenuation, besides induction of alginate (Rau *et al.*, 2010, Garrett *et al.*, 1999, Wu *et al.*, 2004, Govan & Deretic, 1996). Mutation of the gene *lasR* also plays important roles in turning down the virulence while also gaining certain metabolic functions (D'Argenio *et al.*, 2007, Hoffman *et al.*, 2009). RpoN which has been drawing less attention than the other two regulators in CF study, governs the transcription of large amount of genes responsible for virulence, motility, nitrogen and carbon metabolism. *RpoN* mutants were also reported to be isolated from CF patients and a defective *rpoN* gene was responsible for reduced swimming motility and resistance to nonopsonic phagocytosis in some of the isolates (Mahenthiralingam *et al.*, 1994, Smith et al., 2006).

By exploring the unfinished genome sequence drafts of 3 isolates p7-1991, p7-2007 and wtb, we found all the 3 genes have mutations or deletions in p7-2007 and p7-1991 isolates compared to wtb sequence. There is a single nucleotide deletion causing a frame shift in *mucA* gene, which most likely leads to loss of function of MucA protein. The fact that p7-1991 and p7-2007 strains are non-mucoid must be the consequence of reverting from a mucoid origin (*mucA* negative) at certain unknown sites. This reversion is not due to the change of K_{19}→E in AlgU since it also exists in the b mucoid strains. Gene *rpoN* in this strain has a single nucleotide substitution causing an amino acid change L_{419}→P, located in the DNA binding domain which is proximal to DNA in the promoter/holoenzyme complex. This mutation is also expected to have negative impact on RpoN function since it causes a change from non-polar amino acid to a polar one in such an important domain. The third gene *lasR* (PA4130) is completely missing because p7-2007 and p7-1991 strains have a ~5kb gap (referred to Del1) that totally removes gene PA1426- PA1431 and partially PA1425.

To characterize the impact of the *rpoN* mutation, we complemented *rpoN* with a wild type copy amplified from wtb strain using a low copy plasmid pMe6031. We tested the swimming motility and metabolism by Biolog Phenotype Chip. The result showed that complementation can restore 22-25% of the swimming motility measured by swimming zone in soft agar plates (**Figure S2**). Moreover, it also recovered various catabolic functions that RpoN regulates, especially utilization of alternative nitrogen sources, described in later text (**Figure 4**). All these results have suggested that L_{419}→P mutation caused at least partial malfunction of RpoN in p7-1991 and p7-2007 isolates.

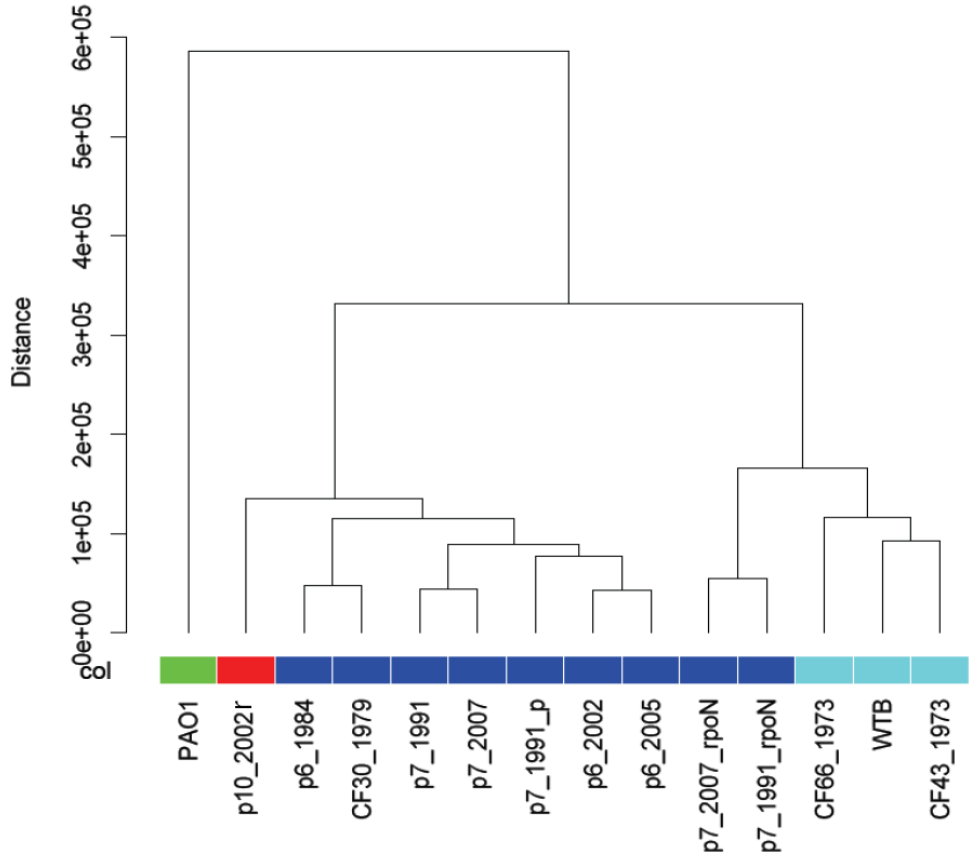

Figure 4. Dendrogram illustrating the relations of various strains based on Biolog Phenotype array data. Late b isolates (dark blue) and p10-2002 r (red) are distant from early b isolates (light blue) and PAO1 (green). When complemented with wild type copies of *rpoN*, p7_1991 and p7-2007 jumped to the same cluster with early b strains (p7_1991_rpoN and p7_2007_rpoN). P7_1991_p is the control where p7_1991 was only complemented with the empty plasmid.

If these 3 mutations are overall beneficial, they should be kept in b population over time in the Copenhagen CF clinic. We then tracked back these 3 mutations in other isolates with a focus on the early ones. The mutations in *mucA* and *rpoN* were tested by sequencing and *lasR* deletion was examined by PCR (described in Materials

and Methods). All the 4 isolates from 1973 have mutations in *mucA* gene, but only strain CF66-1973 has the same one as the 29 later b isolates that we checked. CF66-1973 also has the L_{419}→P mutation identical to 7 later isolates. The *lasR* deletion Del1 first occurred in CF30-1979 isolate and remained in all the later b strains (**Table S2**). Taken together, all 3 mutations in *mucA*, *rpoN* and *lasR* are fixed in the b population in over 40 patients through transmission. Mutations in *mucA* and *rpoN* already existed in CF66-1973 and this strain seems to be the clinical ancestor for all the b isolates. Del1 deletion that removed *lasR* gene appeared in CF30-1979 for the first time. This isolate is also the first b strain that picked up all 3 important mutations, which correlates well with the observation that it already has very similar expression pattern with all the other late isolates but distinct from 1973 isolates. The stability of the 3 mutations in the b population points towards that they are favourable in the adaptation.

To further evaluate the impact of the mutations in the 3 key regulators on the overall transcriptome changes of the late b isolates, we compared the expression data of CF30-1979 which carried the 3 mutations but very few other changes with the environmental representative wtb. We performed a student ttest and extracted the genes with p-value<0.01 and 786 genes were significantly different. We conducted similar analysis on a set of expression data of an intermittent CF isolate (mucA -) and its isogenic mucA+ pair from a previous study which was done under the same condition as ours (Rau et al., 2010). There were 1976 genes significantly changed due to the *mucA* mutation using the same statistical criteria. Between these two data sets, 307 genes overlapped with the same trend in terms of up or down regulation. This means genes regulated by *mucA* mutation are highly represented in CF30-1979 isolate compared to wtb (Fisher's exact test p-value = $1.1e^{-6}$). In previous investigations, gene sets regulated by RpoN and LasR were also determined. Due to the lack of raw data of these two data sets, we could only use the filtered gene lists which involved cut-off of certain fold change. Thus these two data sets might have underestimated the gene numbers regulated by the two genes, comparing to the analysis with no threshold of fold change. There were 62 genes regulated by RpoN (Dasgupta *et al.*, 2003) and 35 genes governed by LasR when cells were harvested at OD_{600} = 0.5 in LB medium (Schuster *et al.*, 2003). There were 21 and 22 genes respectively overlapping with 786 significant gene comparing CF30-1979 and wtb. Again, the genes regulated by *rpoN* and *lasR* are significantly represented in the CF30-1979 vs wtb comparison (Fisher's exact test p-value = 0.02014 and $5.775e^{-5}$ respectively). Nineteen out of 21 genes that are controlled by RpoN are also in the list of mucA- vs mucA+ comparison. In total, among 786 significant genes comparing CF30-1979 against wtb, 331 genes are because

of the mutations in 3 genes *mucA*, *rpoN* and *lasR*, which is 42% of total number (**Figure 5**).

We further assessed the effect of *rpoN* mutation on the metabolic profiles using biolog phenotype chip, since RpoN controls the metabolism of many carbon and nitrogen sources. We selected PM1, PM2 which are the carbon sources plates and PM3 which is the nitrogen plate for our assays. The area underneath the kinetic curves was used for analysis. Instead of focusing on changes of using specific substrates, we applied a hierarchical cluster analysis using data from all three plates (288 wells, 159 informative), to have an overview of the effect of RpoN shown in Figure 4. Similarly with the expression data, separately from PAO1, all the late b clones (after 1979) are clustered together and separated from 1973 isolates and wtb. The separation of the two major clusters is because late isolates have reduced utilization of many substrates. However, p7-1991 and p7-2007 complemented with the wild type *rpoN* gene grouped together with the 1973 isolates. This figure has at least three implications. Foremost, it confirmed that the L_{419}→P mutation is significant causing a reduction of the RpoN function. Additionally, relationships between different strains based on the metabolic profiles agreed with what was concluded from transcriptome data. More importantly, this strongly supports our hypothesis that the *rpoN* mutation has a significant impact on global changes in a variety of phenotypes and it is a huge step ahead in the adaptation process for *P. aeruginosa*.

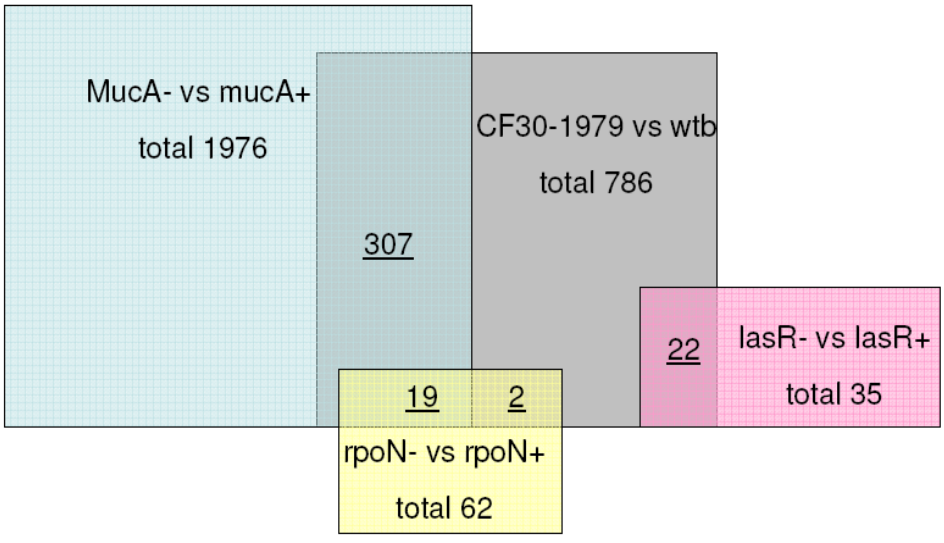

Figure 5. Visualization of impact of *mucA*, *lasR* and *rpoN* mutations on the overall transcriptional profiles of adapted b isolates. The numbers with underlines indicate the gene counts that overlap between the two comparisons.

In conclusion, both transcriptome and catabolic profiles have presented that the b clone experienced substantial evolutionary events from early 1970s to 1979 but remained more constant until 2007, despite that the strains have persisted for much longer time after 1979. Detailed analysis suggested that mutations 3 global regulators *mucA, rpoN* and *lasR* exerted essential control over this transition.

3.3.5 Observation of parallel evolution

So far we have described a dominant clone evolving in parallel in different patients generating a homogenous picture of the adapted *P. aeruginosa* clones. Most likely this is because the ancestor clone picked up important mutations very early which offered great advantage in the adaptation and enables the strain to spread easily in different patients. Thus it is not so called 'parallel evolution' by definition. Here we present an interesting observation which clearly showed b strains have developed the parallel traits independently during the evolution in individuals.

In the genome of P7-2007 strain, we found a ~8 kb gap removing genes from PA4117 to PA4186 and partially PA4116 and PA4187, which is referred to Del2 in present study (**Figure 6a**). This region comprises one operon PA4120 –PA4128 (referred to *hpc* operon) which is responsible for degradation of p-Hydroxy-Phenylacetic Acid (4-HPA) (Zeng & Jin, 2003). 4-HPA is first converted to homoprotocatechuate (HPC) by products of *hpaA* and *hpaC* which are intact in p7-2007 strain. HPC is degraded through a 7 step pathway by enzymes encoded by *hpc* operon into pyruvate and succinic semialdehyde (SSH), which eventually enters TCA cycle (Zeng & Jin, 2003) (**Figure 6b**). The lack of *hpc* operon leads to a deficiency of 4-HPA catabolism, which is proved by the fact that p7-2007 strain could not grow in minimal medium using 4-HPA as a carbon source. We further checked if other strains carry this gap using a simple PCR (see materials and methods) and it turned out around 1/3 of all the b strains have the same deletion as in P7-2007, including earlier isolates from p7 (**Table S2**). This indicates Del2 deletion might have some selective advantage. So we further examined the capability of more strains in utilization of 4-HPA as carbon source. Surprisingly the capacity of degrading 4-HPA was largely reduced or lost in all the strains isolated after year 2000 and showed decline over time in patients p6, p10, CF89 and CF168 (**Table S2**), suggesting a strong selection against catabolism of 4-HPA. The first appearance of impaired growth on 4-HPA is in isolate CF30-1979, which is quite early in the evolutionary process of the b clone.

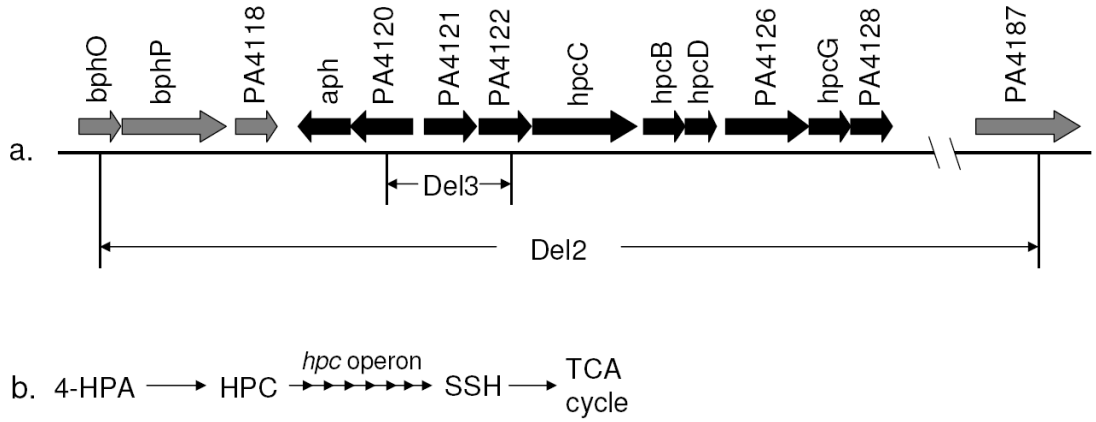

Figure 6. Parallel evolution of deficient *hpc* operon. a) The position of *hpc* operon in the genome and location of two gaps (Del2 and Del3) that lead to impaired *hpc* genes. b) The degradation pathway of 4-HPA and HPC.

To uncover the genetic nature of reduced growth on 4-HPA in those b isolates which do not carry Del2, we designed 5 sets of primers covering most of the *hpc* operon to look for possible small deletions. A ~2kb gap (Del3) was identified in p6-2002 and p6-2005 that causes full deletion of PA4121 as well as partial deletion of PA4122 and PA4120, which is the transcriptional regulator of the *hpc* operon (**Figure 6a**) (Zeng & Jin, 2003). Del3 was also found in CF224-2002, but not in other isolates. When we performed PCR using different primers in this region, we noticed that other patterns of gaps apart from Del2 and Del3 might exist in some strains, but further characterization was not carried out. For those with no apparent deletions such as CF-1979 and P6-1991, we sequenced PA4120 gene but no non-synonymous mutation was found.

Here we did not attempt to identify all the causes of this phenotype in gene levels; what we can show is that the b genotype has developed the same trait – lost or reduced the ability catabolising 4-HPA, through distinct genetic paths, two of which were mapped out (**Figure 6**). This suggests that different lines must have evolved independently in individuals, which is a clear example of parallel evolution occurring in different patients. The evolutionary role of this trait is not known yet, but it must be beneficial for the b clone to persist in CF lungs since it was found repeatedly and separately.

3.4 Discussion

Experimental evolution with microorganisms allows us to observe evolutionary process in real time and understand natural selection in the genetic level. The genetic

basis underlining the evolutionary dynamics has been elucidated in simple defined conditions, but never been evaluated in a complicated and changing natural environment. Our study for the first time demonstrated the process of microbial evolution in a real-world situation – *P. aeruginosa* chronic infection in CF airways.

Utilizing historical collection of *P. aeruginosa* CF isolates belonging to one dominant genotype b in the Copenhagen CF clinic, we estimated the evolutionary paths of *P. aeruginosa* adaptation in CF lung infection and determined some essential steps. An environmental b clone emerged in the CF clinic some time before 1973 and started to persist in CF airways through genetic adaptation. The b clone accumulated beneficial mutations in 3 global regulators by 1979, which significantly enhanced the adaptation and enabled it to transmit and dominate in many patients until now. This generated parallelism in the portraits of all late isolates in terms of transcriptome and metabolic profiles (**Figure 7**). The late isolates have been so severely remodelled during the evolution that they can not be identified as *P. aeruginosa* any more using standard phenotypic based diagnostic methods, indicating genetic adaptation has changed *P. aeruginosa* in the direction of creating a new ecotype which is a specialist thriving in CF airways.

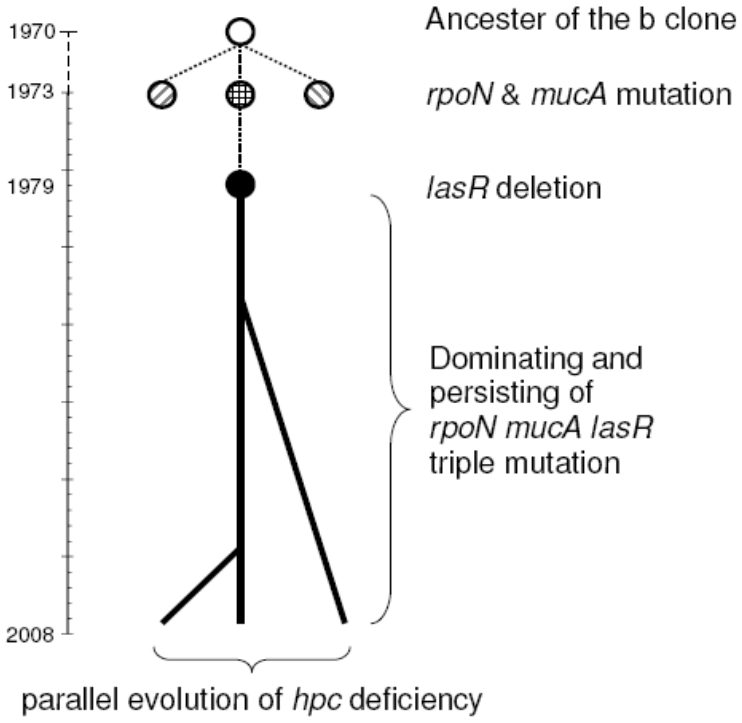

Figure 7. Model of development of important mutations and deletions of the b clone.

Dynamics of evolutionary adaptation revealed by several experimental evolution studies with bacteria and viruses have a common feature that fitness increase is rapid initially but decelerates over time (Cooper & Lenski, 2000, Novella *et al.*, 1995). Due to the fact that there is no good model mimicking the human lung environment, we can not directly measure fitness of different *P. aeruginosa* isolates in CF airways or regenerate their evolutionary trajectory. However, global expression and metabolic profiles as well as other phenotypes (growth rate, motility, *etc*.) of sequential isolates exhibited a strong time dependent progression which could reflect the adaptation process: major changes happened before 1979 and the overall picture remained relatively stable in different patients afterwards for almost 30 years. This observation highly resembles the conclusion drawn from experimental evolution investigations where fitness can be directly quantified.

Typically, early improvement of the fitness is achieved through few mutations with large benefits, rather than accumulations of many mutations with small benefits (Elena & Lenski, 2003). These are often in the regulatory genes with global impact on the expression of many genes. The b cell line successfully persisted probably owning to the early mutations in the 3 regulators *mucA, lasR* and *rpoN*. Mutation in *mucA* gene could activate the alternative sigma factor AlgU, leading to a hallmark phenotype of many CF isolates – mucoid colonies due to massive alginate production, which is believed to be profitable for *P. aeruginosa* to survive in CF lungs (Govan & Deretic, 1996). However more and more evidences implied the importance of pleiotropic effects of AlgT such as reduction of motility, QS and virulence in *Caenorhabditis elegans* model, as well as stress responses, and most of them are independent of alginate production (Rau et al., 2010, Wu et al., 2004). *P. aeruginosa* with *lasR* mutation and QS deficiency are often seen in CF isolates. LasR governs the QS signalling and also has global impact on the secreted virulence factors such as protease. RpoN is also an alternative sigma factor that regulates metabolism of many carbon and nitrogen sources as well as pili and flagella biosynthesis (Dasgupta et al., 2003, Hobbs *et al.*, 1993). RpoN knockout *P. aeruginosa* has dramatically decreased killing in various virulence models (Hendrickson *et al.*, 2001). Apparently, all these 3 regulators have functions in reduction of conventional 'virulence'. In consistency with previous phenotypic characterizations, virulence factors that facilitate pathogens to invade or injure the hosts are very often eliminated during the chronic infection, as opposed to acute infections. Immune evasion is a great advantage in chronic infection of CF patients probably due to better protection from host immune attacks as well as better preservation of host tissue for continuous nutrients supply.

The pleiotropic effects of *rpoN* mutation were further assessed using Biolog phenotype microarrays. In general all the chronic b strains isolated after 1979 have a decay of catabolic potential on almost all the substrates which separates them from those from 1973 and wtb (**Figure S1**). This decay is also the major reason of loss of identity on Biolog ID plates. Figure 4 clearly showed that this decline and parallelism of the decline is mainly because of the *rpoN* mutation. It is counterintuitive that *P. aeruginosa* actually have impaired catabolism on so many carbon and nitrogen sources in minimal medium conditions including amino acids which are very rich in the CF airways. One explanation is that decrease of motility and other virulence factors are the beneficial effects of *rpoN* mutation while decrease of catabolic functions are the evolutionary trade-offs. Cooper et al showed that populations evolved in glucose experienced reduction of catabolic functions of unused substrates and this occurred early and parallel in many independent lines (Cooper & Lenski, 2000). These observations suggested that this specialization is mainly contributed by antagonistic pleiotropy, which is the side effect of early mutations in global regulators. If the decay of catabolic functions that we see on Biolog phenotypic plates is due to the antagonistic pleiotropy of *rpoN* mutation, there are at least two possibilities for the causes. First, *P. aeruginosa* adaptation in CF lungs creates nutritional specialists and their preferred substrates are not included in the tests we performed. For example, there have been indications that fatty acids from airway surface liquid might be suitable carbon sources (Son *et al.*, 2007), they are not present in Biolog Phenotype Plates. Second, it might also be relevant that nutrition utilization is not an important selective factor since the substrates availability is not constrained in CF airways. At least total amino acids can reach very high level in CF sputum samples (Palmer, 2009, Palmer *et al.*, 2007). Even though those chronic isolates do not behave well in minimal media of different compounds, but in a rich media like CF sputa their growth is sufficiently active to support the regeneration and competition. Besides antagonistic pleiotropy, we can also not exclude the possibility that the reduction of catabolic functions is indeed beneficial itself. We have reported before that in the Copenhagen CF isolates many late chronic *P. aeruginosa* isolates with different genotypes have very similar growth rate in various media, which is 2-3 fold reduced compared to PAO1 (Yang et al., 2008). The partially impaired metabolic profiles might provide an optimal growth rate which has proper biomass increase but does not induce too much immune response. Further understanding of this phenomenon requires detailed investigation of more *P. aeruginosa* population and nutrients availability in CF airways.

Parallel evolution typically appeared in a way that same genes were targeted through distinct changes in nucleotide level independently in different patients (Philippe *et al.*, 2007). Parallel evolution is prevalent in nature and is strong evidence that similarities are derived from natural selection rather than random process. Apart from the consistency caused by the 3 pleiotropic mutations, we also described a case story of typical parallel evolution – deficiency of HPC pathway derived from two distinct deletions in the gene operon. Although the role of this phenotype in the evolution is not clear and still under investigation, appearance of parallel changes across multiple lineages strongly indicates that it is adaptive. We further tested 4 randomly picked r strains isolated from 2002 to 2009 and 3 of them can grow well on 4-HPA. Lacking of parallelism of r strains in this trait suggests impaired HPC pathway might be beneficial only in the b genotype and it is probably related to historical contingency of the b evolution.

Besides the widespread of parallelism, we also observed diversity. For example, among the 4 isolates in 1973, there are 3 types of *mucA* sequences. But this diversity was eliminated later and only one type persisted because of the following beneficial mutations, which reflected a transient step of polymorphism during the selective sweep. Another example is the heterogeneity of antibiotic resistance patterns of different strains, which is most likely the consequence of individual therapies received by the patients. Some of the causes have been investigated in p7 isolates. Mutations in *pmrB* and *mexZ* have been found in p7-1991 and p7-2007 compared to wtb, which might explain the resistance of these two strains towards colistin and tobramycin. In contrast to mutations in *mucA*, *lasR* and *rpoN*, these mutations have more 'local' impact on the expression network and respond to specific changes in the micro-environments, which is another level of genetic adaptation in the evolution.

3.6 Conclusion and future directions

The human airways are a heterogeneous and dynamic environment which is expected to generate diversities during *P. aeruginosa* evolution. However, the high levels of parallelism that we observed might reflect large extent of ecological homology in the lung environment, probably supported by coughing and treatments decreasing the mucus viscosity. It could also be because that the stresses from immune attack are very similar in different zones of the CF airways and thus eliminating the pathogenic display is the dominating trait evolved during *P. aeruginosa* adaptation. Nevertheless, the evolution of the b clones generated a consistent pattern in different patients

because early mutations that were highly adaptive made them successfully sweep across many patients and persisted. The end result of the evolution is a generalist capable of occupying all the niches in CF airway. However, we can not predict if this will be applicable to other *P. aeruginosa* infections in CF patients. The evolutionary dynamics of r genotype is right now under investigation to obtain a more completed picture of *P. aeruginosa* adaptation. Other driving forces other than random mutation, such as acquisition of novel genetic elements which could highly accelerate the evolution are also being studied by completion and careful inspection of the genome sequences.

3.7 Materials and Methods

3.7.1 Strains and growth conditions

P. aeruginosa isolates were selected by plating sputum samples directly on Pseudomonas Isolation Agar (PIA) plates containing 100 μg/ml of ampicillin. Single colonies were cultivated and stored at -80°C with glycerol. The genotype of *P. aeruginosa* isolates was identified using either ***Pulsed Field Gel Electrophoresis*** (PFGE) or AT biochips (Clondiag Chip Technologies, Germany). All strains were cultured in Luria Broth (LB) at 37 °C unless otherwise noted.

3.7.2 Sample preparation and array processing of DNA microarrays

For transcriptome analysis, all *P. aeruginosa* strains were grown aerobically at 37 °C in LB medium starting from OD_{600} = 0.01 and harvested at OD_{600} = 0.5. Samples were prepared according to the prokaryotic sample and array processing protocol from Affymetrix (Santa Clara, CA). Harvested cells were mixed immediately with RNAprotect Bacteria Reagent (Qiagen) and stored at -80 °C. RNA was extracted with RNeasy mini kit (Qiagen) and transcribed to cDNA using SuperScript III Reverse Transcriptase (Invitrogen). The cDNA was purified with Qiagen PCR purification kit (Qiagen) and fragmented using DNase I (Promega). Fragmented cDNA was then labelled using labelling reagent (Affymetrix) and Terminal Deoxynucleotidyl Transferase (Promega). The labelled cDNA was hybridized on Affymetrix *P. aeruginosa* PAO1 gene chip and stained on GeneChip® Fluidics Station 450. The probe arrays were finally scanned with GeneChip® Scanner 3000. The raw data (.cel files) were obtained using the Affymetrix GeneChip® Operating System 1.4 (GCOS).

3.7.3 Transcriptome data analysis

Transcriptome data analysis was performed using BioConductor tools in R environment (www.bioconductor.org). Basic data processing including normalization and expression index calculation was done with RMA package (Bolstad *et al.*, 2003). The principle component analysis was conducted with *svd* function. Pair-wise comparison by student t-test was performed using function *t.test*.

3.7.4 Sequencing of *lasR, mucA, algT* and *rpoN* genes

Sequencing of genes *lasR, mucA, algT* and *rpoN* was performed by PCR using High Fidelity DNA Polymerase (Fermentas) and four sets of primers: *lasR* fwd (5′- TCTCGGACTGCCGTACAAC -3′) and *lasR* rev (5′- AATTACCGATCGCCAGCTC –3′) for *lasR* gene, *mucA* fwd (5′- CTCTGCAGCCTTTGTTGCGAGAAG -3′) and *mucA* rev (5′- CTGCCAAGCAAAAGCAACAGGGAGG -3′) for *mucA* gene, *algT* fwd (5′- CCTG AGCCCGATGCAATCCATTTTCG -3′) and *algT* rev (5′- GGACAGAGTTTCCTGCA GGGCTTCAC -3′) for *algT* gene, *rpoN* fwd (5′- CTCGCCAACGACCTGGTCAAG -3′) and *rpoN* rev (5′- GGCGTCGGTCACATCCAGTTG -3′) for *rpoN* gene. Another two primers were used for *rpoN* sequencing, *rpoN* seq1 (5′- GCCTGGGAAGACATCTA C CAGAC -3′) and *rpoN* seq2 (5′- GAGCTGAACCAGGAGGCGATG -3′).

3.7.5 Verifying deletion regions

The primers used for verifying the deletion region PA1425-1429 are del1fwd (5′- AAAGGAATCCAGCATGCACATTCG -3′) and del1rev (5′- AGCCTTTCTGTCCAG AGTTGATGG -3′). The strain carrying the deletion can produce a PCR fragment of 732bp, while the wild type like strains will have a 5kb product. The primers to confirm the deletion region PA4116-4187 are del2fwd (5′- CCCAGGGCCTGAAGGA AGTG -3′) and del2rev (5′- CTGCTGCAGGTTCAGCGAAC -3′). PCR on strains with this deletion can give a band of 912bp and those do not have the deletion have no PCR products. To confirm that the latter one is not false negative, one gene in this region (PA4125) was amplified using primers set PA4125fwd (5′- GGCAGATCAAC GCGGTATT -3′) and PA4125rev (5′- GTTCAGGGTGCTGGCTGT -3′). The primers to verify the deletion region of PA4120-4122 are del3fwd (5′- GGTAGCCGGCAAGGTA GT -3′) and del3rev (5′-AGGGTCATGAAGCTGGAGAA-3′). These primers generate a 579bp DNA band those have this region deleted and a 2.5kb fragment was produced otherwise.

3.7.6 RpoN complementation

A wild type copy of *rpoN* gene was amplified from wtb strain using primers *rpoN* NcoI fwd (5′- ATGCCATGGTGAAAACGGCGTATATCGTCA -3′) and *rpoN* EcoRI rev (5′- CCGGAATTCGTTGATGGCCACTGATGTTG -3′). The PCR fragment was digested with NcoI and EcoRI and ligated into pMe6031 vector (Heeb *et al.*, 2000) creating plasmid pLYA. The plasmid pLYA was introduced into different strains by conjugation using the helper strain pRK600. Transformants were selected on PIA plates containing 60 μg/ml of tetracycline.

3.7.7 Biolog Phenotype Microarray™

Phenotype MicroArrays (Biolog, Hayward, CA) was performed in duplicates according to the manufacturer's instructions. *P. aeruginosa* strains were streaked on LB agar plates and incubated at 37 °C until colonies appeared on the plates (16-30 h). Cells were swabbed from the plates and suspended in IF-0 GN Base (inoculation fluid) at a density corresponding to 42 % transmittance in the Biolog turbidimeter. The cell suspensions were diluted 1:6 in IF-0 minimal medium containing Biolog redox dye mixture D (tetrazolium), and 100 μl aliquots were added to carbon source plates (PM1 and PM2). For the nitrogen source plate (PM3), inoculations were supplemented with 30 mM glucose and 2 μM of ferric citrate. The plates were incubated at 37 °C in an OmniLog plate reader, and growth/respiration was measured kinetically by determining the colorimetric reduction of tetrazolium dye. Of the total 285 substrates available on the three PM plates, 159 were found informative (supporting consistent growth between replicates for at least one strain and not autoinducing the color change). Export of OmniLog data was performed using OmniLog *OL_FM/Kin* 1.20.02 software (Biolog). Average area underneath each kinetic curve is used for analysis. Hierarchical clustering analysis was performed in R with the package "*squash*" (A. C. Eklund).

3.7.8 OmniLog Identification System

OmniLog Identification System (Biolog) was used for test the identification of various strains. Assays were performed according to manufacture's protocols except the incubation time was extended to 3 days due to the slow growth of certain strains. Identification was done in an automated manner by comparing the array pattern with Biolog Database. 3 strains were tested: wtb, p7-2007 (b) and p2-2005 (r). Strain wtb

was identified as *P. aeruginosa*, p7-2007 was only identified at genus level and p2-2005 was not even identified at genus level.

3.7.9 Phenotypic assays

Motility: The twitching, swimming and swarming motilities were assayed on ABT plates (Lei's paper ref 9) supplemented with 0.5% glucose and 0.5% casamino acid. 1.5%, 0.3% and 0.6% of agar was used for twitching, swimming and swarming plates respectively. Bacterial cells were stabbed in the bottom, in the middle and on top of the plates for twitching, swimming and swarming motility, respectively. Twitching plates were incubated for 48 hours at 37 °C and the agar was removed to measure the twitching zone on the petri dishes. The swimming zones were measured after 24 hours incubation at 30 °C, while the swarming zones were measured after 24-48 hours incubation at 37 °C. PAO1 knockout mutant ΔpilA (Klausen *et al.*, 2003) was used as a negative control for twitching motility and ΔfliM (Klausen et al., 2003) was used as a negative control of swimming and swarming motility.

Minimal Inhibitory Concentrations: Minimal Inhibitory Concentration (MIC) values of ciprofloxacin, tobramycin, aztreonam and colistin were measured using Etest® strips (AB biodisk, Sweden) by plating 100µl of overnight culture on LB plates. The values were recorded after 48 h incubation in 37 °C.

Quorum sensing: Quorum sensing signal production were tested by streaking isolates and two *E. coli* monitor strains harbouring lasB-gfp or rhlA-gfp fusion (Hentzer *et al.*, 2002) that can be induced by *las* signal molecule (3-O-C_{12}-HSL) and *rhl* signal molecule (C_4-HSL), respectively. After 24 h of incubation at 37 °C, GFP production was visualized using a Zeiss Axion2 microscope with an integrated Coolsnap color cf camera at 2.5× Magnification and an exposure time of 200ms. Slow growing strains were incubated 8 hours before the monitor strains were applied to make sure that they are in stationary phase after 24 hours.

Hypermutability: Hypermutability was determined by mutation frequencies of rifampicin and streptomycin as described previously (Ciofu *et al.*, 2008). The strain is considered to be hypermutable if it has 20 times higher mutation frequency than PAO1.

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3.8 Supplementary materials

Table S1 Phenotypes of selected strains

CF No.	DTU No.	Isolation Year	Sample No.	Doubling Time (min)	las	rhl	Swimming	Swarming	Twitching	CO	TM	CI	AZ
			PAO1	24	++	++	++++	++++	++++	7	2	0.19	1
510	B54	2007	wtb	27	++	++	++++	++++	++++	6	2	1	3.5
43		1973	206	34	++	++	++++	++++	++++	1.5	1	0.5	>256
43		1984	19319A	41	++	++	++++	-	+	3	6	0.38	>256
43		1985	20155	61	++	++	++	-	+	1.5	3	1	>256
114		1973	218	26	++	++	++++	++++	++++	1	0.19	0.094	>256
105		1973	208	33	-	-	++++	-	+	3	0.5	0.38	>256
66		1973	217	50	++	+	+	-	-	3	1.5	0.75	64
66		1992	20538	62	-	-	+	-	-	3	24	8	>256
66		2008	5561A	56	-	-	+	-	-	6	24	12	>256
66		2008	5561B *	90	-	-	+	-	-	0.38	16	24	>256
30		1979	1912	51	-	-	+	-	-	1.75	4	0.32	>256
173	p6	1984	18198	63	-	-	+	-	-	4	10	0.32	>256
173	p6	2002	76863A	83	-	-	+	-	-	8	9	4	>256
173	p6	2005	s1C2	72	-	-	+	-	-	7	5	5.5	>256
333	p7	1991	14429	56	-	-	+	-	-	7	7	1	96
333	p7	1997	15281	59	-	-	+	-	-	>256	9	1	>256
333	p7	2003	5896A	74	-	-	+	-	-	>256	19	6	>256
333	p7	2005	S1B1	76	-	-	+	-	-	>256	24	4	>256
333	p7	2007	0apr	78	-	-	+	-	-	>256	26	3	>256
333	p7	2007	apr *	109	-	-	+	-	-	>256	21	2	>256
243	p10	2002	75425E	110	-	-	-	-	-	1.75	16	>32	>256
243	p10	2002	75425F r	61	-	-	-	-	-	1.75	16	>32	>256

CO: colistin; TM: tobramycin; CI: ciprofloxacin; AZ: aztreonam. MIC values (μg/ml) were measured using E-test strips. Doubling times were measured in LB culture with shaking at 37°C. r: 'r' genotype, *: mucoid

Table S2 characterization of strains and patients

Patient No.	Isolation Year	sample No.	*mucA*	*algT*	*lasR*	*rpoN*	PA4116-4187 region	Growth on 4-HPA
		PAO1	wt	wt	wt	wt	wt	wt
CF510	2007	wtb	wt	wt	wt	wt	wt	wt
CF 43	1973	206	t_{352}->c,118stop	g_{349}->t, 117stop	wt	wt	n.d.	wt
CF 43	1984	19319A	t_{352}->c & g_{354}->t, Q_{118}->Y	wt			n.d.	wt
CF 43	1985	20155		wt			n.d.	wt
CF 114	1973	218	c_{382}->t,128stop	c_{448}->g, R_{150}->G	wt	wt	n.d.	wt
CF 105	1973	208	t_{352}->c,118stop	c_{248}->a, A_{83}->E	wt	wt	n.d.	wt
CF 66	1973	217	430Δg & frame shift	g_{55}->a, K_{19}->E; 116 insertion & frame shift	g_{647}->a, R_{224}->G	t_{1256}->c, L_{419}→P	n.d.	wt
CF 66	1992	20538	430Δg & frame shift		Del1		Del2	No
CF 66	2008	5561A	430Δg & frame shift		Del1		Del2	No
CF 66	2008	5561B	430Δg & frame shift		Del1		Del2	No
CF 30	1979	1912	430Δg & frame shift		Del1	t_{1256}->c,L_{419}→P	n.d.	p
P2	1991	14495	430Δg & frame shift		Del1		Del2	
P2	1994	15286b	430Δg & frame shift		Del1		n.d.	
P2	2005	S1F6 r			wt	t_{652}->c,C_{218}→R; g_{842}->a,G_{281}→D	n.d.	
P7	1991	14429	430Δg & frame shift		Del1	t_{1256}->c,L_{419}→P	Del2	No
P7	1997	15281			Del1		Del2	No
P7	2003	5896A			Del1		Del2	No
P7	2004	34243			Del1		Del2	No
P7	2005	S1B1	430Δg & frame shift	g_{55}->a, K_{19}->E	Del1		Del2	No
P7	2007	Apr-00	430Δg & frame shift	g_{55}->a, K_{19}->E	Del1	t_{1256}->c,L_{419}→P	Del2	No
P7	2007	07apr-3*	430Δg & frame shift	g_{55}->a, K_{19}->E	Del1	t_{1256}->c,L_{419}→P	Del2	No

P7	2007	07Nov*	430Δg & frame shift	wt	Del1		Del2	No
p7	2008	07feb*		g_{55}->a, K_{19}->E	Del1		Del2	No
P6	1984	18198	430Δg & frame shift		Del1	t_{1256}->c,L_{419}→P	n.d.	wt
P6	1991	11026	430Δg & frame shift		Del1		n.d.	P
P6	2002	76863A	430Δg & frame shift		Del1	t_{1256}->c,L_{419}→P	Del3	No
P6	2005	S1C2	430Δg & frame shift		Del1	t_{1256}->c,L_{419}→P	Del3	No
P10	1986	100	430Δg & frame shift		Del1		n.d.	P
P10	1991	15806	430Δg & frame shift		Del1		n.d.	P
P10	2002	75425E	430Δg& frame shift		Del1		n.d.	P
P10	2002	75425F r			t_{530} ->c, L_{176} →P	wt	n.d.	
P11	2003	1299b	430Δg & frame shift		Del1		Del2	No
P11	2005	S1F3	430Δg & frame shift		Del1		Del2	No
P16	1985	48A	430Δg & frame shift		Del1		n.d.	
P16	1987	22125			Del1		n.d.	P
P16	1991	10318A	430Δg & frame shift		Del1		n.d.	P
P16	2002	7329B*	430Δg & frame shift	g_{55}->a, K_{19}->E	Del1		n.d.	No
CF 211	1991	15563	430Δg & frame shift		Del1		Del2	No
CF 211	1997	50171a*	430Δg & frame shift		Del1		Del2	No
CF 211	1997	50171b	430Δg & frame shift		Del1		Del2	No
CF 211	2006	A11*	430Δg & frame shift		Del1		Del2	No
CF 211	2006	F8/06	430Δg & frame shift		Del1		Del2	No
CF 223	2002	2937C*			Del1		Del3	No
CF 223	2002	2937B			Del1		Del3	No
CF 131	2001	79740A			Del1		n.d.	No
CF 224	2002	2268A*			Del1		n.d.	No
CF 224	2002	2268C			Del1		n.d.	No
CF 222	2001	81819C			Del1		n.d.	No
CF 206	2002	3741A			Del1		Del2	No
CF 206	2002	3741B*			Del1		Del2	No

CF 253	2002	2846C*	Del1	n.d.	P
CF 240	2002	13049B*	Del1	Del#	P
CF 240	2002	13049A	Del1	Del#	P
CF 180	2002	2943B	Del1	Del#	No
CF 180	2002	2943C*	Del1	Del#	No
CF 248	2002	44336	Del1	Del2	No
CF 311	2002	14676B	Del1	n.d.	No
CF 311	2002	14676C	Del1	n.d.	No
CF 249	2002	51833	Del1	Del2	No
CF 205	2002	2149B*	Del1	Del2	No
CF 205	2002	2149A	Del1	Del2	No
CF 178	2002	6286a	Del1	n.d.	No
CF 124	2001	77666B	Del1	Del2	No
CF 231	2002	570A	Del1	n.d.	No
CF 126	2001	73236A*	Del1	n.d.	No
CF 126	2001	73236B	Del1	n.d.	No
CF 263	2001	79676B*	Del1	n.d.	No
CF 153	2001	82664B	Del1	n.d.	No
CF 153	2002	81461B	Del1	n.d.	No
CF 155	2001	74931A*	Del1	Del2	No
CF 216	2002	6370A*	Del1	n.d.	P
CF 216	2002	6370B	Del1	Del2	No
CF 273	2002	8730/ 8731	Del1	Del2	No
CF 202	2002	9518B	Del1	n.d.	No
CF 229	2002	4945A*	Del1	n.d.	No
CF 229	2002	4945B	Del1	n.d.	No
CF 160	2002	4765	Del1	n.d.	No
CF 47	1991	14958	Del1	n.d.	No
CF 47	1995	11392A	Del1	Del2	No

CF 61	1984	19696	Del1	Del2	No
CF 61	1999	68000D	Del1	Del2	No
CF 21	1985	1738B	Del1	n.d.	No
CF 59	1991	14765B	Del1	n.d.	No
CF 59	1994	14724	Del1	n.d.	No
CF 89	1984	19234	Del1	n.d.	wt
CF 89	1995	6253A	Del1	n.d.	No
CF 168	1984	20688B	Del1	n.d.	wt
CF 168	1995	5284B	Del1	n.d.	No

1. Del: deletion; wt: wild type, identical or with silent mutations compared to PAO1; n.d.: not totally deleted, but not known whether the region is identical to wt; *, mucoid phenotype; r: 'r' genotype, besides the two strains marked 'r' and PAO1, all the other strains are b genotype; Blank: not checked
2. Mutations are marked first with change in nucleotide level (small letters) and then with amino acid level (capital letters). Two different mutations in amino acid level in same gene are separated with ' ; '.
3. Highlighting colors: green, mutations are located in important domains; orange, mutations are not located in any known domains.
4. Growth on 4-HPA was categorized into 3 types. No: no growth; wt: wild type growth; p: reduced growth

Table S3 Genes that contribute most to the first SVD

ORF	Gene Name	Regulation #	Product name
PA0045		down	hyperthetical protein
PA0046		down	hyperthetical protein
PA0047		down	hyperthetical protein
PA0062		up	hyperthetical protein
PA0120		up	probable transcriptional regulator
PA0121		up	hyperthetical protein
PA0165		down	hyperthetical protein
PA0277		down	conserved hyperthetical protein
PA0291	*oprE*	down	Anaerobically-induced outer membrane porin OprE precursor
PA0329		up	conserved hyperthetical protein
PA0365		up	hyperthetical protein
PA0366		up	probable aldehyde dehydrogenase
PA0588		up	conserved hyperthetical protein
PA0745		up	probable enoyl-CoA hydratase/isomerase
PA0762	*algU*	up	sigma factor algU
PA0763	*mucA*	up	anti-sigma factor
PA0807	*ampDh3*	up	AmpDh3
PA1077	*flgB*	down	flagellar basal-body rod protein FlgB
PA1078	*flgC*	down	flagellar basal-body rod protein FlgC
PA1079	*flgD*	down	flagellar basal-body rod modification protein FlgD
PA1080	*flgE*	down	flagellar hook protein FlgE
PA1082	*flgG*	down	flagellar basal-body rod protein FlgG
PA1092	*fliC*	down	flagellin type B
PA1093		down	hypothetical protein
PA1094	*fliD*	down	flagellar capping protein FliD
PA1095		down	hypothetical protein
PA1096		down	hypothetical protein
PA1183	*dctA*	up	C4-dicarboxylate transport protein
PA1228		down	hyperthetical protein
PA1323		up	hyperthetical protein
PA1324		up	hyperthetical protein
PA1431	*rsaL*	down	regulatory protein RsaL
PA1432	*lasI*	down	autoinducer synthesis protein LasI
PA1471		up	hyperthetical protein
PA1552		down	probable cytochrome c
PA1554		down	probable cytochrome oxidase subunit (cbb3-type)
PA1559		up	hyperthetical protein
PA1560		up	hyperthetical protein
PA1592		up	hyperthetical protein
PA1632	*kdpF*	up	KdpF protein
PA1985	*pqqA*	up	pyrroloquinoline quinone biosynthesis protein A
PA1999	*dhcA*	up	DhcA, dehydrocarnitine CoA transferase, subunit A

PA2014	*liuB*	up	methylcrotonyl-CoA carboxylase, beta-subunit
PA2015	*liuA*	up	putative isovaleryl-CoA dehydrogenase
PA2016	*liuR*	up	regulator of liu genes
PA2018	*mexY*	up	Resistance-Nodulation-Cell Division (RND) multidrug efflux transporter
PA2019	*mexX*	up	Resistance-Nodulation-Cell Division (RND) multidrug efflux membrane fusion protein precursor
PA2247	*bkdA1*	up	2-oxoisovalerate dehydrogenase (alpha subunit)
PA2248	*bkdA2*	up	2-oxoisovalerate dehydrogenase (beta subunit)
PA2252		down	probable AGCS sodium/alanine/glycine symporter
PA2317		up	probable oxidoreductase
PA2381		up	hyperthetical protein
PA2445	*gcvP2*	down	glycine cleavage system protein P2
PA2446	*gcvH2*	down	glycine cleavage system protein H2
PA2485		up	hyperthetical protein
PA2562		up	hyperthetical protein
PA2634	*aceA*	up	isocitrate lyase AceA
PA2779		up	hyperthetical protein
PA3031		up	hyperthetical protein
PA3038		up	probable porin
PA3049	*rmf*	up	ribosome modulation factor
PA3147	*wbpJ*	down	probable glycosyl transferase WbpJ
PA3148	*wpbI*	down	probable UDP-N-acetylglucosamine 2-epimerase WbpI
PA3151	*hisF2*	down	imidazoleglycerol-phosphate synthase, cyclase subunit
PA3155	*wbpE*	down	probable aminotransferase WbpE
PA3158	*wbpB*	down	probable oxidoreductase WpbB
PA3159	*wbpA*	down	probable UDP-glucose/GDP-mannose dehydrogenase WbpA
PA3179		down	conserved hyperthetical protein
PA3229		up	hyperthetical protein
PA3452	*mqoA*	down	malate:quinone oxidoreductase
PA3552	*arnB*	up	conserved hypothetical protein
PA3553	*arnC*	up	probable glycosyl transferase
PA3554	*arnA*	up	conserved hypothetical protein
PA3555	*arnD*	up	conserved hypothetical protein
PA3556	*arnT*	up	inner membrane L-Ara4N transferase ArnT
PA3557	*arnE*	up	conserved hypothetical protein
PA3558	*arnF*	up	hypothetical protein
PA3559		up	probable nucleotide sugar dehydrogenase
PA3602		up	conserved hyperthetical protein
PA3610	*potD*	down	polyamine transport protein PotD
PA3691		up	hyperthetical protein
PA3692		up	probable outer membrane protein precursor
PA3722		down	hyperthetical protein

PA3819		up	conserved hyperthetical protein
PA3904		down	hyperthetical protein
PA4070		up	probable transcriptional regulator
PA4073		up	probable aldehyde dehydrogenase
PA4101		up	probable two-component response regulator
PA4102		up	probable two-component sensor
PA4103		up	hypothetical protein
PA4104		up	conserved hypothetical protein
PA4105		up	hypothetical protein
PA4106		up	conserved hypothetical protein
PA4107		up	hypothetical protein
PA4110	*ampC*	up	beta-lactamase precursor
PA4111		up	hyperthetical protein
PA4220		up	hyperthetical protein
PA4359		up	conserved hyperthetical protein
PA4525	*pilA*	down	type 4 fimbrial precursor PilA
PA4607		up	hyperthetical protein
PA4645		down	probable purine/pyrimidine phosphoribosyl transferase
PA4773		up	hyperthetical protein
PA4774		up	hyperthetical protein
PA4775		up	hyperthetical protein
PA4776	*pmrA*	up	PmrA: two-component regulator system response regulator PmrA
PA4777	*pmrB*	up	PmrB: two-component regulator system signal sensor kinase PmrB
PA4781		up	probable two-component response regulator
PA4782		up	hypothetical protein
PA4880		up	probable bacterioferritin
PA5015	*aceE*	down	pyruvate dehydrogenase
PA5016	*aceF*	down	dihydrolipoamide acetyltransferase
PA5117	*typA*	down	regulatory protein TypA
PA5178		up	conserved hypothetical protein
PA5182		up	hyperthetical protein
PA5192	*pckA*	down	phosphoenolpyruvate carboxykinase
PA5212		up	hyperthetical protein
PA5298		down	xanthine phosphoribosyltransferase

When the regulation is 'down', the expression level of the gene decreases from right to left on the first SVD in Figure 2, and it is generally down regulated when infection progresses; vice versa. This table was co-contributed by Jens Vindahl Kringelum, Rasmus Lykke Marvig, Julie Serritslev, Mikael Holm Thomsen and Juliane Charlotte Thøgersen.

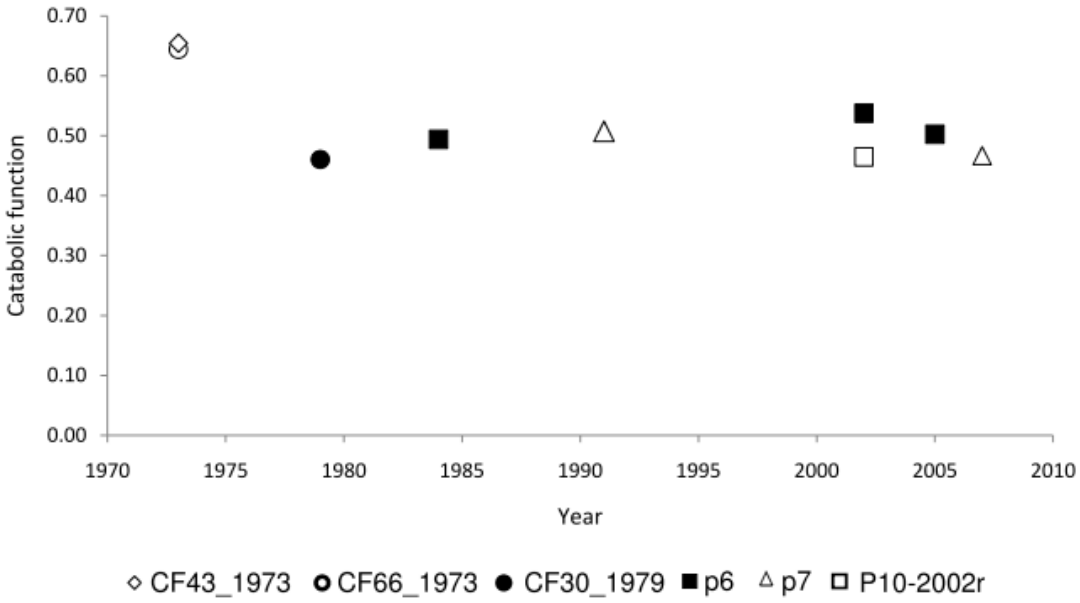

Figure S1. Catabolic functions of b isolates. The catabolic function of each isolate is calculated as the average value of all the 159 wells relative to PAO1. Isolates after 1979 have a significant decay compared to those from 1973.

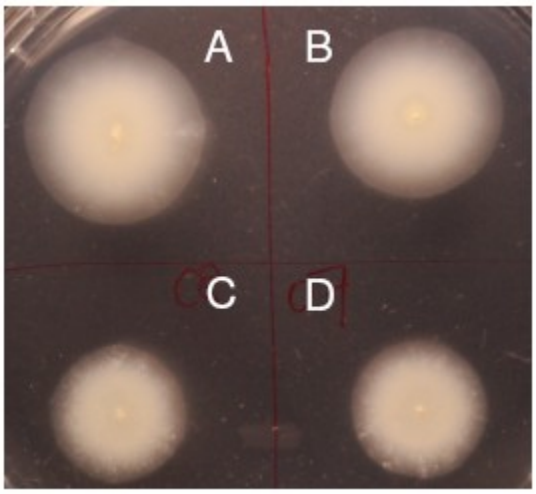

Figure S2. Swimming zones of A and B) p7-2007 complemented with wild type *rpoN* carrying vector; C) p7-2007; D) p7-2007 plus the vector without wild type *rpoN*. Diameters: A) 16mm; B) 16mm; C) 13mm; D) 12mm.

Chapter 4 Conclusions, Discussions and Future Directions

4.1 The longest microbial evolution 'experiment' creating a new ecotype

P. aeruginosa persistence in chronic CF patients became the focus of our study not only due to its obvious clinical significance but also because it is an excellent model system to understand the microbial evolution in general and bacterial adaptation when switching from the environment to human lungs.

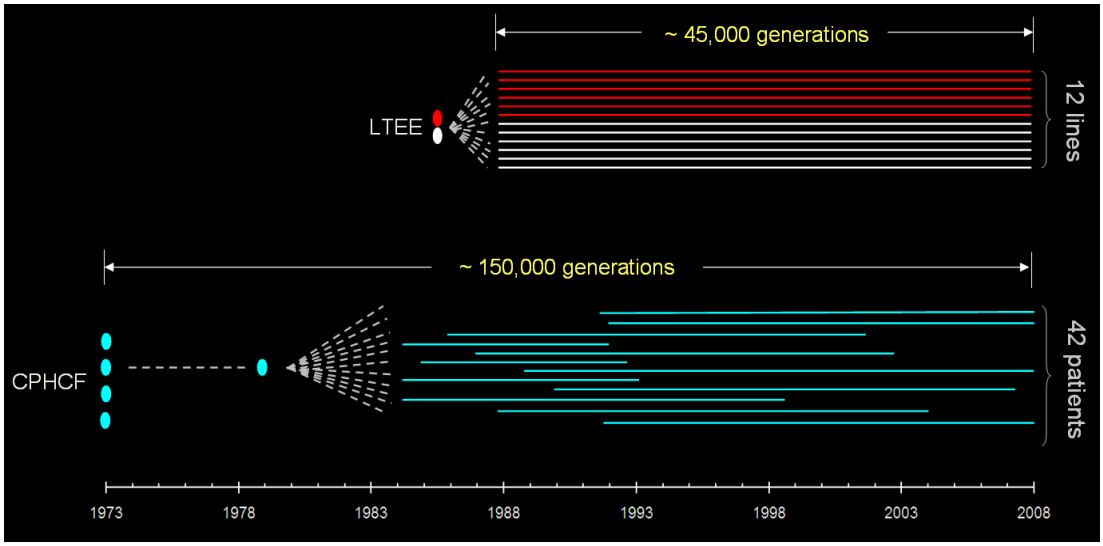

Figure 1. Overview of strain dynamics in the LTEE setting (adapted from http://myxo.css.msu.edu/index.html) and the b clone development in Copenhagen CF centre (CPHCF).

The unique 'fossil' collection of *P. aeruginosa* isolates from Copenhagen CF centre since 1973 allows us to perform the paleontological study of the b clone. Using FISH we could determine the *in situ* growth rates of *P. aeruginosa* in chronic CF lungs, which is around 115 min (doubling time) on average. This allows us to estimate the number of generations from 1973 to 2008. The b clone has adapted for about 150,000 generations in a group of 42 patients since 1973, which makes it the longest microbial evolution 'experiment' ever reported (**Figure 1**). Moreover, this project is to study the

genetic changes from a long term adaptation in complex natural environments (CF lungs) instead of defined lab conditions, which is to our knowledge the first ever seen.

If we make a portrait of the well adapted *P. aeruginosa*, we realize that evolution has shaped the bacterium so deeply that we could hardly recognize it is a *P. aeruginosa*. They have reduced growth rate in various lab conditions, decline in the production of classical virulence factors and decreased capacity of metabolizing different substrates. They even have no identities on Biolog Microbial Identification chips. Compared to environmental isolates, they are literally 'losers'. However, inside CF airways they are able to out-compete environmental invaders. Thus, evolution has created a niche specialist of *P. aeruginosa* that can only thrive in CF lung environment where the stresses from immune attack and antibiotics are severe, with a trade-off of decreased fitness in other environments, such as simple lab media.

In Lenski's *E.coli* long term experiment, citrate was added in their experiment to inspect contamination because *E. coli* species can not grow on citrate. However, a novel variant that could utilize citrate derived from certain historical contingency during the evolution. They brought up the discussion whether this variant will eventually become distinct species (Blount *et al.*, 2008). One problem in bacterial systematics is that the definition of species is theory-free and only based on empirical criteria using molecular or genomic approaches (Cohan & Perry, 2007). Usually two members with in the species share over 70% homology in their genome content or 99% homology in 16sRNA. In our study, identities of the late b isolates were lost when they were tested based on metabolic profiles, but maintained in more conserved assays, such as FISH with probes targeting 16sRNA. Since mutation accumulation increases linearly over time (Barrick *et al.*, 2009) (although late mutations might not give rise to profound phenotypic changes), we would expect given enough time they could eventually become two species distinct in genome content – one major in reproducing in natural environment and the other specializing to live in human CF airways. Most likely the evolution of b clone in CF patients is on the track of creating new species. So far the concept of 'ecotype' seems to be better describing the stage of the adapted b clones. Ecotype defines a group of bacteria that are ecologically similar to one another (Cohan & Perry, 2007). Long term genetic adaptation of *P. aeruginosa* in CF patients generated an ecotype that is dedicated to survival in CF airways and ecologically distant from their environmental relatives.

4.2 Evolutionary paths: Genetic adaptation and more

Despite the fact that we could not directly measure fitness of various *P. aeruginosa* isolates in CF airway, we can still obtain an approximation of the evolutionary dynamics of the b clone adaptation based on transcriptional and metabolic changes. Most likely fitness gain was profound by 1979 and much slower afterwards until now. The early fast fitness increase was mainly due to mutations in 3 regulatory genes *lasR*, *mucA* and *rpoN*. They are adaptive most likely through down-regulation of various virulence factors. The also have side effects due to their global regulatory networks. For example mutation in *rpoN* led to impaired metabolic profiles. It would be interesting to find out if there are compensatory mutations that could restore some pleiotropic effects of previous adaptive mutations, which give rise to small benefits.

Besides the b clone, there is another dominant clone belonging to the r genotype. It would be appealing to investigate if it follows the same dynamic as the b clone, and if the same beneficial pleiotropic mutations occurred early as well. There are also strains that are able to persist in one patient but inadequate to transmit, which are also interesting to study. All these investigations will give us a more complete picture of evolution dynamics of *P. aeruginosa* adaptation in CF patients.

Our study has described how a *P. aeruginosa* clone went through genetic changes (mutations and deletions) from an environmental strain to a strain fully adapted to the lung, which is very much comparable to isolated pure culture evolving in a constant condition. However, different microbes in patient airways are not isolated from each other and there could be horizontal gene transfer among bacteria. Various genes associated to virulence or antibiotics resistance can be carried by mobile genetic elements (plasmids, phages, transposons and integrons) or variable regions of the chromosome (e.g. pathogenicity islands). They are horizontally transferred by means of transformation, conjugation or transduction. Gene gaining by horizontal gene transfer could dramatically speed up the evolutionary process. These traits belonging to so called 'evolution by acquisition' have not been inspected in the present study.

The CF lung environment is a dynamic and complex system which should favour diversity. So far we have described a homogenous picture of b clone evolution due to the 3 highly adaptive mutations it 'luckily' picked up very early. But in certain functions or conditions, there could be diversities formed for various reasons, such as spatial heterogeneity due to the difference in micro-environments in the lung, diversity maintained by cooperation and cheating, phenotypic variation determined by fluctuating environments, polymorphism caused by high mutation rates, *etc*. The

next phase of our studies will have a focus on the diversity occurring during the adaptation and compared it with the dynamic of the b clone.

Considering that CF lung is like an open 'playground' with many species to co-exist, the interaction and parallel evolution of different microorganisms is an attractive to study. These might have affected the evolutionary dynamics of each individual species.

Besides, the differences among individuals in terms of immune responses or airway conditions could lead to distinct evolutionary progresses of *P. aeruginosa*. However, this subject itself is extremely complicated and so far we can only assume all patients are more or less the same. The change from environment to human body is much larger and dominant in driving the evolution.

In conclusion, *P. aeruginosa* adaptation in CF patients could be a consequence of different elements combined and some of them are more important than others. The efforts that we make to understand the bacteria adaptation mechanism will eventually help us discover new solutions to prevent or cure persistent infections.

4.3 Parasitic symbiosis: Is pathogenesis an evolutionary 'mistake'?

Although in this thesis we focused attention on interpreting the evolutionary process of *P. aeruginosa* adaptation to the CF airways, eventually we hope to obtain better understanding of pathogenesis of *P. aeruginosa* infection and find improved therapeutic solution for CF patients. We have presented a model describing *P. aeruginosa* evolution in chronic CF patients through mutations. Early mutations in three important genes *lasR*, *mucA* and *rpoN* all play roles in reduction of classical virulent factors (**Figure 2**), suggesting that the traditional view of virulence is not applicable in chronic infection of *P. aeruginosa* in CF patients. We have mentioned in **section 1.1.1** that *P. aeruginosa* can cause both acute infections like bacteraemia killing the host in days and chronic infection that can last for decades. What makes them to decide whether to damage the host using virulent factors or to drop their 'weapons' and have a steady life in the host? How should we define virulence in such chronic infection?

To answer this question, we need look beyond Pseudomonas and learn from other symbiosis relationships. *Mycobacterium tuberculosis* for instance, the predominant cause of airway infection tuberculosis can persist in human airways by maintaining a dormant form (also see 1.3.4) and patients do not exhibit clinical evidence of active

disease. *M. tuberculosis* in this form is non-culturable and insusceptible to drugs. However, when the immune system is suppressed (e.g., co-infection with HIV), *M. tuberculosis* can escape immune surveillance and establish overt disease. But it is very common that after chemotherapy *M. tuberculosis* relapses into the quiescent state and persists (Trucksis, 2000). The *M. tuberculosis* persistence is a parasitic symbiosis in a fine-tuned fashion that has evolved over time between the bacterium and host. Evasion of immune responses by reducing antigenic presentation during persistence has also been observed in *Salmonella enteria serovar* Typhi and *H. pylori* long-term inhabitation in individuals who do not develop overt diseases (Monack *et al*., 2004). Similarly, *P. aeruginosa* is also trying to be invisible for host immune response, by attenuating toxin production through gradual genome alterations but keeping relatively high growth rate.

What is the role of 'virulence factors' for bacteria in the context of co-evolution between microbes and their eukaryotic neighbours for millions of years? If we compare parasitic symbiosis with mutualistic (beneficial) symbiosis, we can see a striking homology in the apparatuses that microbes use to 'damage' or 'help' the host. For example, in the beneficial association between *Vibro fischeri* and monocentrid fishes, *V. fischeri* uses QS to control transcription in high cell densities, whereas QS has been considered as one of the major toxins in *P. aeruginosa*. A type III secretion system was found to be critical to transfer of the bacterial cells into the host bacteriocytes in a mutualistic symbiosis between grain weevils and their bacterial endosymbionts (Dale *et al*., 2002), while type III secretion system is recognized as a key virulence factor in many bacterial pathogens. McFall-Ngai and Gordon presented a view about this convergence of the beneficial and pathogenic interactions. If symbiotic evolution is driven by selection of organisms that can thrive through the interaction of their counterparts, beneficial symbiosis should be the rule rather than the exception, while pathogens may have perverted their relationships with their hosts (McFall-Ngai & Gordon, 2006). In this sense, it is counterproductive for pathogens to destroy their hosts, which are their sources of nutrition. Pathogenesis might be an evolutionary 'mistake' in which the microbe-host alliance fall apart (Kolter & Hogan, 2006).

If we come back to the story of *P. aeruginosa*, how can we interpret the long-term association between *P. aeruginosa* and human? Can we consider the adaptation of *P. aeruginosa* through loss of virulence as actions to correct this 'mistake'? The fact that *P. aeruginosa* can persist better than other pathogens in CF lungs might not be because

they are better to invade, but they are better to compromise. *P. aeruginosa* is rather a 'victim' than a 'winner' in the interaction with their host – CF patients. Being silent might the best way to survive in a host then beneficial symbiosis can not be established. Compared to bacteraemia that can destroy the host very fast, chronic infection is better for reproductive success in the long run because it preserves their colonizing niches. The driving force of establish chronic or acute infection is to large extend on the side of the host, including how strong immune response is and how efficient the chemotherapies are.

Based on limited data available, we could speculate the following model giving a novel view of pathogenesis: microbial infection happens when the microorganism enters a 'wrong' host where beneficial association can not be established. In case of acute infection, pathogens are either cleared out quickly by antibiotics or immune defence or causing mortality of the host. Either way, there are no steady relationship between pathogens and the host. In case of chronic infection, the host suppression (including both immune defences and antibiotic treatment) is probably moderate, neither too strong to eradicate the bugs, nor too weak to be overwhelmed by the microbial proliferation. Thus pathogens have time and are selected to adjust their dialogs with the host, by switching growth mode or genetic adaptation or other mechanisms, and a relative long-term equilibrium can be sustained between the two.

Finally, it is essential to know that *P. aeruginosa* infection in CF patients are constantly active, and there is no latent phase when patients experience no symptoms at all as in *M. tuberculosis* persistence. Besides passively removing the antigenic molecules, *P. aeruginosa* have properties that help the bacteria actively defend themselves against the host attack and explore nutrients or important substrates, which we suggest should be referred to the real 'virulence factors' for such kind of chronic infection, including antibiotic resistance, stress response triggered by AlgT activation, and other traits yet to be identified.

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